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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. 1.53 (c)

Docket Number	1662.026PRV	Type a plus sign (+) inside this box >	+

INVENTOR(s)/APPLICANT(s)

Name (last, first, middle initial)	RESIDENCE (CITY, AND EITHER STATE OR FOREIGN COUNTRY)
Gladwin, Mark T. Cannon, III, Richard	Washington, DC Potomac, MD

TITLE OF THE INVENTION (280 characters max)

METHOD FOR TREATMENT OF CARDIOVASCULAR MALCONDITIONS USING NITRIDES

CORRESPONDENCE ADDRESS

Schwegman, Lundberg, Woessner & Kluth
P. O. Box 2938
Minneapolis, Minnesota 55402

Attn: Peter L. Malen

STATE	Minnesota	ZIP CODE	55402	COUNTRY	United States of America

ENCLOSED APPLICATION PARTS (check all that apply)

XXX	Specification	Number of Pages	29		Small Entity Statement
XXX	Drawing(s)	Number of Sheets	26		Other (specify)

METHOD OF PAYMENT (check one)

	A check or money order is enclosed to cover the Provisional filing fees	PROVISIONAL FILING FEE AMOUNT	$160.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No.

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Respectfully submitted,

SIGNATURE _____ Date July 9, 2003

TYPED OR PRINTED NAME Peter L. Malen REGISTRATION NO. 44,894

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re **PROVISIONAL** Patent Application of: Mark T. Gladwin et al.

Title: METHOD FOR TREATMENT OF CARDIOVASCULAR MALCONDITIONS USING NITRIDES

Docket No.: 1662.026PRV

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SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
P.O. Box 2938, Minneapolis, MN 55402 (612-373-6900)

By: _____
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(NEW FILING)

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UNITED STATES PROVISIONAL PATENT APPLICATION

METHOD FOR TREATMENT OF CARDIOVASCULAR MALCONDITIONS USING NITRITES

Inventors: MARK T. GLADWIN, a citizen of the United States of America, residing at 4927 Nebraska Avenue, NW in Washington, DC, 20008; and

RICHARD O. CANNON, III, a citizen of the United States of America, residing at 9413 Kentsdale Drive in Potomac, MD, 20854.

Assignee: The Government of the United States of America,
as represented by the Secretary of the Department of Health and Human Services,
6011 Executive Blvd., Suite 325,
Rockville, Maryland 20852

Prepared and filed by:

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
1600 TCF Tower
121 South Eighth Street
Minneapolis, Minnesota 55402

METHOD FOR TREATMENT OF CARDIOVASCULAR MALCONDITIONS USING NITRITES

Statement of Government Rights

The invention was made with the support of a grant from the Government of the United States of America (Clinical Center and NHLBI intramural NIH funds and NIH grant HL58091). The Government may have certain rights to the invention.

Background of the Invention

At high concentrations nitrite is a vasodilator *in vitro*.[1-6] *In vivo* plasma levels of nitrite range from 150 to 1000 nM, and the concentration in aortic ring tissue is in excess of 10 μM.[7-9] This potential storage pool for NO is in vast excess of plasma S-nitrosothiols, now reported to be less than 10 nM in human plasma.[9-12] Mechanisms have been proposed for the *in vivo* conversion of nitrite to NO either by enzymatic reduction by xanthine oxidoreductase or by non-enzymatic disproportionation/acidic reduction.[13-21] Both mechanisms would occur preferentially in vascular regions with low pH and low partial pressure of oxygen. Indeed, consistent with oxygen and pH sensitive chemistry, hypoxia and acidosis potentiate NO generation and vasodilation from both nitrite and NO donors in aortic ring bioassay and lung perfusion bioassay systems.[22-24] However, in a recent study , Kelm and colleagues infused nitrite into the forearm circulation of three human subjects for one minute and reported no vasodilatory effects.[25] This report indicated that under physiological conditions nitrite would not function as an intravascular storage pool for NO and thus as an intrinsic vasodilator.

Consistent with the bioconversion of intravascular nitrite to NO, arterial-to-venous gradients of nitrite across the human forearm at rest and during regional NO synthase inhibition have been observed, with increased consumption of nitrite occurring with exercise.[8,26] Kelm and colleagues have also shown that large artery-to-vein gradients of nitrite form across the human forearm during NO synthase inhibition.[25] Unlike the more simple case of oxygen extraction across a vascular bed, nitrite is both consumed, as evidenced by artery-to-vein gradients during NO

synthase inhibition and exercise, and produced in the vascular bed by endothelial NOS-derived NO reactions with oxygen. Supporting the existence of an intravascular NO species capable of storage and distal delivery of NO bioactivity, it has been observed that red blood cells and plasma "loaded" with NO, by exposure to NO solutions, NO gas or NO donors can export NO and induce vasodilation *in vitro* and *in vivo*.[11,27-32]

Therefore, there is a need for further knowledge of pathways and systems that regulate NO production, for example, for designing treatments to regulate a patient's blood pressure.

Summary of the Invention

These and other needs are met by the current invention. It has been discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. This discovery provides useful treatments to regulate a patient's blood pressure, for example, by the administration of nitride salts.

The present invention provides a method for decreasing a patient's blood pressure, including administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

The present invention also provides method for treatment of a patient with a cardiovascular malcondition, including administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

The present invention also provides method for treatment of a patient with a cardiovascular malcondition, including administering to the patient an effective amount of a pharmaceutically-acceptable compound to modulate the nitrite-deoxyhemoglobin-nitric oxide system.

The present invention further provides method to treat or prevent the deleterious ancillary effects of increased blood pressure in a patient, including administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

In some embodiments of the invention, the administration is parenteral, peritoneal, oral, bucal, rectal, *ex vivo*, or intraocular. In some embodiments, the administration is intravenous, intraarterial, subcutaneous or intramuscular.

The present invention also provides a method of diagnosis of a malcondition in a patient, including determining the amount of nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide in a physiological sample from a patient and comparing the level of the nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide to the normal range of nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide in the sample, wherein a difference in the amount of indicative of the presence and/or predictive of the development of the malcondition.

Malconditions associated with elevated blood pressure include but are not limited to stroke, heart disease, kidney disease and failure, eye damage including hypertensive retinopathy, diabetes, and migraines.

Nitrite anions are present in concentrations of about 150-1000 nM in the plasma and about 10 μM in aortic tissue. This represents the largest vascular storage pool of nitric oxide (NO), provided physiological mechanisms exist to reduce nitrite to NO. The vasodilator properties of nitrite in the human forearm and the mechanisms extant for its bioactivation were investigated. Sodium nitrite was infused at about 36 μmoles per minute into the forearm brachial artery of 18 normal volunteers, resulting in a regional nitrite concentration of about 222 μM and an immediate about 175% increase in resting forearm blood flow. Increased blood flow was observed at rest, during NO synthase inhibition and with exercise, and resulted in increased tissue perfusion, as demonstrated by increases in venous hemoglobin-oxygen saturation, partial pressure of oxygen, and pH. Systemic concentrations of nitrite increased to about 16 μM and significantly reduced mean arterial blood pressure. In an additional six subjects, the dose of nitrite was reduced about 2-logs resulting in a forearm nitrite concentration of about 2 μM and an about 22% increase in blood flow. Nitrite infusions were associated with the formation of erythrocyte iron-nitrosyl-hemoglobin, and to a lesser extent, S-nitroso-hemoglobin

across the forearm vasculature. The formation of NO-modified hemoglobin appears to result from the nitrite reductase activity of deoxyhemoglobin, linking tissue hypoxia and nitrite bioactivation. These results indicate that physiological levels of blood and tissue nitrite represent a major bioavailable pool of NO that contributes to vaso-regulation and provides a mechanism for hypoxic vasodilation via reaction of vascular nitrite with deoxygenated heme proteins. Substantial blood flow effects of nitrite infusion into the brachial artery of normal human subjects results from forearm nitrite concentrations as low as about 1.5 μM.

Brief Description of the Figures

Figure 1. Hemodynamic and metabolic measurements at baseline and during exercise, without (A) and with (B) inhibition of NO synthesis in 18 subjects. Mean arterial pressure (MAP), forearm blood flow (FBF), and venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2), and pH are shown for all experimental conditions. These interventions and measurements (part I of the protocol) served as a control for part II of the protocol, in which these interventions were performed during nitrite infusion.

Figure 2. Effects of infusion of sodium nitrite in bicarbonate-buffered normal saline into the brachial arteries of 18 healthy subjects at baseline and during exercise, without (A) and with (B) inhibition of NO synthesis. Values for mean arterial blood pressure (MAP), forearm blood flow (FBF), venous oxyhemoglobin saturation, partial pressure of oxygen (pO_2) and pH, venous nitrite, venous iron-nitrosyl-hemoglobin and venous methemoglobin are shown for all experimental interventions.

Figure 3. Formation of iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin following nitrite infusion. Panel A: NO levels (mV signal) from arterial and venous blood hemoglobin measured by ozone-based chemiluminescence. Samples incubated without mercury (-$HgCl_2$) represent total iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin and samples incubated with mercury (+$HgCl_2$) represent only iron-nitrosyl-hemoglobin. The difference in peak area is S-nitroso-hemoglobin. The levels of iron-nitrosyl-hemoglobin (Panel B) and S-nitroso-hemoglobin (Panel

C) increased from artery to vein, consistent with formation across the vascular bed following nitrite infusion. The levels increased most during exercise despite the fact that venous nitrite levels decreased with exercise. Panel B, inset: Arterial blood EPR spectra were subtracted from venous blood EPR spectra, demonstrating an increase in iron-nitrosyl-hemoglobin from artery to vein. Difference spectra from three patients during exercise with nitrite infusion are shown. Panel D: Formation of iron-nitrosyl-hemoglobin (black squares) and S-nitroso-hemoglobin (red circles) during nitrite infusion at baseline, during nitrite infusion and during nitrite infusion with exercise, quantified by subtraction of the arterial from the venous levels and multiplying the result by blood flow. Asterix signify $P<0.05$ by paired t test or repeated measures analysis of variance.

Figure 4. The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation in both *in vivo* and *in vitro* experiments. Panel A: *In vitro* incubations of 200 μM nitrite with erythrocytes from 0-100% oxygen saturation at room temperature for 10 minutes demonstrated an inverse relationship between iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin formation and hemoglobin oxygen saturation. Panel B: A similar inverse relationship was observed in the human circulation during nitrite infusion (for iron-nitrosyl-hemoglobin $r=-0.7$, $p<0.0001$, for S-nitroso-hemoglobin $r=-0.45$, $p=0.04$). Hemoglobin oxygen saturation was measured from the antecubital vein by co-oximetry.

Figure 5. Model for the erythrocyte as a hemoglobin-nitrite reductase system participating in oxygen-dependent NO homeostasis. Nitrite is taken up by erythrocytes through the anion exchange protein (AE1 or Band 3) or through the membrane as nitrous acid (a pH dependent process that would further accelerate nitrite reduction during hypoxia). The AE1 protein, by binding both deoxyhemoglobin and methemoglobin, localizes catalytic NO and S-nitrosothiol generation from nitrite to the erythrocyte membrane. NO formed from nitrite reaction with deoxyhemoglobin, and NO_2 formed from the nitrite-methemoglobin (or nitrite-oxyhemoglobin) reaction react with each other ($NO + NO_2$) at diffusion limits to form N_2O_3. N_2O_3 generated at the membrane could directly nitrosate

glutathione, with rapid export of low molecular weight S-nitrosothiol by simple diffusion across the erythrocyte membrane. Potential export of NO, NO_2, and nitrated lipids must also be considered. Similar chemistry could occur directly at the endothelium or smooth muscle with deoxy-globins and other heme proteins.

Figure 6. Diagram of some physiological effects of NO.

Figure 7. Diagram of mechanism of NO-mediated smooth muscle cell relaxation.

Figure 8. Diagram of NO involvement.

Figure 9. Vascular effects of endothelium-derived versus hemoglobin-transported NO in healthy subjects.

Figure 10. Forearm measurement system.

Figure 11. NO breathing restores forearm vasodilator tone during inhibition of regional NO synthesis.

Figure 12. Inhaled NO is vasoactive.

Figure 13. NO reactions with oxygen, thiols, and heme.

Figure 14. NO inhalation and NO synthesis experimental results.

Figure 15. NO inhalation and NO synthesis experimental results.

Figure 16. NO physiology.

Figure 17. Previous studies indicate that nitrite lacks vasodilator action in humans.

Figure 18. Nitrite is a source of bioactive NO in human subjects.

Figure 19. Vaso-activity of nitrite infusion.

Figure 20. Formation of NO-Hb adducts during nitrite infusion.

Figure 21. NO-Hb adducts form from reaction of nitrite with deoxyhemoglobin.

Figure 22. Nitrite-hemoglobin chemistry.

Figure 23. Diagram of NO physiology.

Figure 24. Diagram of NO physiology.

Figure 25. Relaxation response to nitrite at high oxygen tension and low oxygen tension.

Figure 26. Exhaled NO during nitrite perfusion in isolated rat lung.

Detailed Description of the Invention

It has been discovered that nitrite is reduced to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. It has further been discovered that administration of pharmaceutically-acceptable salts of nitrite are useful in the regulation of the cardiovascular system. These discoveries provide useful methods to prevent and treat malconditions associated with the cardiovascular system, for example, high blood pressure.

Vasodilatory properties of nitrite *in vivo*

Eighteen healthy subjects (9 males, 9 females; age range 21 to 50 years) were enrolled in a physiological study to determine if nitrite is a vasodilator and to examine nitrite's *in vivo* chemistry. Part I of the protocol measured the normal hemodynamic and metabolic responses to exercise and to inhibition of NO synthesis within the forearm as a control for part II of the protocol, in which these interventions were performed during nitrite infusion. Initial baseline measurements included a mean blood pressure of 85.6 ± 3.7 mm Hg and forearm blood flow of 4.0 ± 0.3 ml/min per 100 mL tissue (Figure 1A). Repetitive hand-grip forearm exercise increased blood flow approximately 600% over resting values, and significantly decreased ipsilateral venous hemoglobin oxygen saturation, pO_2, and pH, consistent with increased oxygen consumption and CO_2 generation. Following a 20-minute rest period, repeat hemodynamic measurements showed an approximate 10% higher forearm blood flow, but no change in systemic blood pressure or forearm venous hemoglobin oxygen saturation, pO_2 and pH values compared with the initial baseline values (Figure 1B). The NO synthase inhibitor L-NMMA was then infused into the brachial artery at 8 μmol/min for 5 minutes, significantly reducing forearm blood flow by approximately 30% and significantly reducing venous hemoglobin oxygen saturation, pO_2 and pH values. Repeat forearm exercise during continued L-NMMA infusion increased blood flow, but to a significantly lower peak value compared with exercise alone ($P<0.001$). In addition, hemoglobin oxygen saturation, pO_2 and pH were significantly lower during exercise with L-NMMA than with exercise

without regional NO synthase inhibition ($P<0.001$, $P<0.005$ and $P=0.027$, respectively). Mean arterial blood pressure was unchanged during all components of Part I of the protocol.

To determine whether nitrite has vasoactivity in humans, in Part II of the protocol sodium nitrite in bicarbonate-buffered normal saline (final concentration 36 μmol/ml) was infused into the brachial arteries of 18 healthy subjects to achieve an estimated intravascular concentration of approximately 200 μM (1 mL of 36 mM nitrite solution infused into 200 mL of blood flowing to the forearm per minute). Following repeat baseline measurements and infusion of sodium nitrite at 1 mL/min for 5 minutes, nitrite levels in the ipsilateral antecubital vein increased from 3.32 ± 0.32 to 221.82 ± 57.59 μM (Figure 2A). Forearm blood flow increased 175% over resting values; venous hemoglobin oxygen saturation, pO_2 and pH levels significantly increased over pre-infusion values, consistent with increased perfusion of the forearm.

Systemic levels of nitrite were 16 μM as measured in the contralateral arm. These levels produced a systemic effect, as mean systemic blood pressure fell by approximately 7 mm Hg. Consistent with immediate NO generation from nitrite, iron-nitrosylated-hemoglobin in the ipsilateral antecubital vein increased from 55.7 ± 11.4 to 693.4 ± 216.9 nM during the nitrite infusion (chemistry described in detail herein). During forearm exercise with continuation of the nitrite infusion, blood flow increased further, with evidence of metabolic stress by virtue of reduction in forearm venous hemoglobin oxygen saturation, pO_2 and pH levels from baseline values. Venous nitrite levels declined, consistent with increased blood flow to the forearm diluting the concentration of infused nitrite. Despite decreasing forearm nitrite concentrations during exercise iron-nitrosyl-hemoglobin levels increased (consistent with an augmented rate of NO generation from nitrite during exercise-discussed below).

Following cessation of nitrite infusion and substitution of saline as the intra-arterial infusate for 30 minutes, repeat baseline measurements showed persistent elevations in systemic levels of nitrite, iron-nitrosyl-hemoglobin and methemoglobin (Figure 2B) over values obtained prior to the infusion of nitrite

almost one hour before. In addition, persistence of a vasodilator effect was also apparent, as forearm blood flow was significantly higher (4.79 ± 0.37 versus 3.94 ± 0.38 mL/min per 100 mL tissue, P=0.003) and systemic blood pressure significantly lower (82.1± 3.7 versus 89.2 ± 3.5 mm Hg, P=0.002) than initial pre-nitrite infusion values. During re-infusion into the brachial artery of sodium nitrite 36 μmol/ml, combined with L-NMMA 8 μmol/min in order to again inhibit regional synthesis of NO, similar vasodilator effects of nitrite on resting and exercise forearm blood flow were seen as during nitrite infusion without L-NMMA (Figure 2B). This stands in contrast to the vasoconstrictor effect of NO synthase inhibition with L-NMMA observed in Part I of the protocol (Figure 2A). Venous nitrite and iron-nitrosyl-hemoglobin levels followed similar patterns during NO inhibition as during the initial nitrite infusion.

As a test of the physiological relevance of vascular nitrite as a vasodilator, the nitrite concentration was reduced 2-logs to 400 nmol/mL. An infusion of 1 mL/min for five minutes in six subjects significantly increased forearm blood flow from 3.69 ± 0.21 to 4.58 ± 0.35 ml/min per 100 mL tissue, P=0.017. Mean venous nitrite levels increased from 154 nM to 2245 nM following a five-minute infusion. These data indicate that basal levels of nitrite, from 150-1000 nM, contribute at least in part to resting vascular tone and hypoxic vasodilation.

The vasodilatory property of nitrite during basal blood flow conditions, when tissue pO_2 and pH are not exceedingly low, was surprising and unexpected. These results indicate that the previously hypothesized mechanisms for nitrite reduction, nitrite disproportionation and xanthine oxidoreductase activity, both of which require extremely low pO_2 and pH values not typically encountered in normal physiology, are complemented *in vivo* by additional factors that serve to catalyze nitrite reduction. While ascorbic acid and other reductants, present in abundance in blood, may provide necessary electrons for nitrous acid reduction, such that the reaction might occur at physiologically attainable pH levels, it is herein reported that deoxyhemoglobin effectively reduces nitrite to NO within one half-circulatory time and provides a graded production of NO tightly regulated by hemoglobin oxygen desaturation.

Intravascular formation of NO and S-nitrosothiol by reaction of nitrite with intraerythrocytic deoxyhemoglobin

Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" and the iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin content determined by tri-iodide-based reductive chemiluminescence and electron paramagnetic resonance spectroscopy as described in Methods. As previously reported,[30] and recently confirmed,[9] the baseline levels of S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were at the limits of detection (<50 nM or 0.0005% NO per heme) with no artery-to-vein gradients. Following nitrite infusion in part II of the protocol, venous levels of both iron-nitrosyl-hemoglobin and S-nitroso-hemoglobin rose strikingly (Figure 3A, B, and C). This formation of iron-nitrosyl-hemoglobin across the forearm circulation was confirmed by electron paramagnetic resonance spectroscopy (Figure B inset). The formation of both NO-hemoglobin adducts occurred across the vascular bed, a half-circulatory time of less than 10 seconds. The rate of NO formation, measured as iron-nitrosyl and S-nitroso-hemoglobin and quantified by subtraction of the arterial from the venous levels and the difference multiplied by blood flow, increased greatly during exercise, despite a significant decrease in the venous concentration of nitrite secondary to increasing blood flow diluting the regional nitrite concentration (Figure 3D; P=0.006 for iron-nitrosyl-hemoglobin and P=0.02 for S-nitroso-hemoglobin by repeated measures ANOVA).

The formation of both NO-hemoglobin adducts was inversely correlated with hemoglobin-oxygen saturation, which fell during exercise stress, measured from the antecubital vein by co-oximetry (for iron-nitrosyl-hemoglobin r=-0.7, P<0.0001, for S-nitroso-hemoglobin r=-0.45, P=0.04); Figure 4B). *In vitro* incubations of 200 μM nitrite with whole blood from 0-100% oxygen saturation for 10 minutes (room temperature) recapitulated the *in vivo* data, indicating that the NO and SNO formation was dependent on the reaction of nitrite with deoxyhemoglobin (Figure 4A).

A mechanism of iron-nitrosyl- and S-nitroso-hemoglobin formation *in vivo*

One of the interesting observations emanating from the study of NO-hemoglobin biochemistry is that protein S-nitrosation occurs in red blood cells, albeit at low levels (<50 nM), despite the presence of high affinity heme sinks for NO.[30,31,34] The mechanisms underlying this S-nitroso-hemoglobin formation have not been established. For example, the autoxidation of NO to yield nitrite and S-nitrosothiol, via the intermediacy of N_2O_3, is exceedingly slow under physiological oxygen concentrations and should be prohibited by vicinal heme groups. Such kinetic constraints would explain the limited formation of S-nitroso-hemoglobin during NO inhalation and the lack of artery-to-vein gradients of S-nitroso-hemoglobin in the human circulation.[29,35] Therefore, the levels of both iron-nitrosyl- and S-nitroso-hemoglobin formed *in vivo* in this study are striking. During a transit time less than 10 seconds through the forearm circulation during exercise, infused nitrite (200 μM regional concentration) produced approximately 750 nM iron-nitrosyl-hemoglobin and 200 nM SNO-Hb. While the reaction of nitrite with deoxyhemoglobin to form NO and iron-nitrosyl-hemoglobin has been well characterized,[33] the observed formation of S-nitroso-hemoglobin is unexpected and indicates new *in vivo* chemistry.

Luschinger and colleagues[36] recently proposed that nitrite reacts with deoxyhemoglobin to make iron-nitrosyl-hemoglobin, as described by Doyle et al.,[33] with subsequent "transfer" of the NO to the cysteine 93 to form S-nitroso-hemoglobin mediated by reoxygenation and quaternary T to R transition of hemoglobin. However, a direct transfer of NO from the heme to the thiol requires NO oxidation to NO+ and such "cycling" has not been reproduced by other research groups.[31] Rather than a transfer of NO to cysteine during hemoglobin reoxygenation, it is herein proposed a direct reaction of nitrite with methemoglobin or oxyhemoglobin to form nitrogen dioxide (NO_2) with a subsequent radical-radical reaction with NO (generated by the nitrite-deoxyhemoglobin reaction) to form N_2O_3, as shown in equation series 1 and 2.

Equation series 1

NO_2^- (nitrite) + $HbFe^{II}$ (deoxyhemoglobin) + $H^+ \rightarrow HbFe^{III}$ (methemoglobin) + NO + OH^-

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow HbFe^{II}NO$ (iron-nitrosyl-hemoglobin)

Nitrite is first reduced to form NO and methemoglobin with a rate constant of 2.9 $M^{-1}sec^{-1}$ (measured at 25°C).[33] This reaction will be pseudo-first order, governed by the vast amounts - 20 mM - of intra-erythrocytic hemoglobin, and limited only by the rate of nitrite uptake by the erythrocyte membrane. NO then either binds to deoxyhemoglobin to form iron-nitrosyl-hemoglobin or reacts with nitrogen dioxide (NO_2) (or a thiyl radical) produced via equation series 2.

Equation series 2

NO_2^- (nitrite) + $HbFe^{III} \rightarrow HbFe^{III}ONO^- \rightarrow HbFe^{II}ONO$ (Fe^{II}-nitrogen dioxide intermediate)

NO (from Equation series 1) + $HbFe^{II}ONO \rightarrow HbFe^{II}ONONO \rightarrow HbFe^{II} + N_2O_3$

N_2O_3 + R-SH $\rightarrow$ R-S-NO

In the second series of reactions, nitrite binds with methemoglobin directly and the electron from nitrite transfers to the metheme via outer sphere electron transfer at 0.3 $M^{-1}sec^{-1}$ to form an Fe^{II}-ONO intermediate.[37] NO formed from the nitrite-deoxyhemoglobin reaction could then react with the nitrogen dioxide (NO_2) in a diffusion-limited radical-radical reaction ($k=10^9$ $M^{-1}sec^{-1}$, that could effectively compete with heme nitrosylation) to form Fe^{II}-ONONO and then N_2O_3, the primary nitrosating nitrogen oxide.[38,39] Neighboring β-globin cysteine 93 or intracellular glutathione would then be nitrosated ($k= 10^{7-8}$ $M^{-1}sec^{-1}$) to form a hemoglobin or glutathione S-nitrosothiol. This provides a novel mechanism for anaerobic S-nitrosothiol formation and overcomes the kinetic implausibility of slow NO oxidation in the presence of hemoglobin, a high affinity NO scavenger. The formation of NO_2 may also result in other competing chemistry within the erythrocyte, such as hydrogen extraction from thiols to form thiyl radicals and

nitration of lipids. Thiyl radicals could form disulfides or react with NO to form S-nitrosothiols.

A mechanism for S-nitrosothiol formation via hemoglobin-nitrogen dioxide (NO_2) chemistry is supported by the anaerobic production of S-nitroso-hemoglobin in our *in vitro* experiments (Figure 4A) and the increasing yield with decreases in hemoglobin oxygen saturation across the forearm *in vivo* (Figure 4B). The alternative possibility proposed by Luschinger and colleagues[36] that iron-nitrosyl-hemoglobin formed by the reaction of nitrite with deoxyhemoglobin with subsequent transfer to cysteine to form S-nitroso-hemoglobin during reoxygenation was evaluated. Nitrite was incubated with erythrocytes anaerobically and then reoxygenated the erythroctes for 30 to 600 seconds and observed no conversion of the iron-nitrosyl-hemoglobin to SNO-Hb following reoxygenation (data not shown). Consistent with the anaerobic S-nitrosothiol forming reaction requiring nitrite reaction with methemoglobin, the addition of cyanide inhibited S-nitroso-hemoglobin formation but not iron-nitrosyl-hemoglobin formation under strict anaerobic glove box conditions (data not shown). Reaction of nitrite with oxyhemoglobin produced no iron-nitrosyl- or S-nitroso-hemoglobin (see 100% oxyhemoglobin saturation values in Figure 4A and 4B).

Physiological considerations

It is demonstrated herein that nitrite produces vasodilation in humans and can be reduced to NO by deoxyhemoglobin. Remarkably, systemic levels of 16 μM, only one log higher than the levels of nitrite present in blood, result in systemic vasodilation and decreased blood pressure, and regional forearm levels of only 2 μM increase blood flow 22%. While not a limitation of the present invention, it is further proposed that *in vivo* chemistry for the conversion of nitrite to NO and S-nitrosothiol by reaction with deoxyhemoglobin and methemoglobin provides a mechanism for hypoxia-regulated catalytic NO production by the erythrocyte or endothelial/tissue heme proteins.

Three factors uniquely position nitrite, rather than S-nitrosothiol, as the major vascular storage pool of NO: 1) Nitrite is present in substantial

concentrations in plasma, erythrocytes and in tissues.[7] 2) Nitrite is relatively stable, because it is not readily reduced by intracellular reductants (as are S-nitrosothiols[30]) and its reaction rate with heme proteins is 10,000 times less than that of authentic NO. 3). Nitrite is only converted to NO by reaction with deoxyhemoglobin (or presumably deoxy-myoglobin, -cytoglobin, and -neuroglobin) and its "leaving group" is the met(ferric)heme protein which will not scavenge and inactivate NO.[33] Therefore, this pool provides the ideal substrate for NO generation during hypoxia, providing a novel mechanism for hypoxic vasodilation.

Because a deoxyhemoglobin-nitrite reductase system would result in NO formation in deoxygenating blood, such a system links hemoglobin oxygenation status to NO generation, the principle previously ascribed to S-nitroso-hemoglobin.[34] Hemoglobin possesses anionic binding cavities that retain nitrite[30] and nitrite is taken up by erythrocytes through the anion exchange protein (AE1 or Band 3) or through the membrane as nitrous acid (a pH dependent process that accelerates nitrite uptake during tissue hypoxia.[40,41] Such nitrite would provide a steady source of NO, NO_2 and S-nitrosothiol generation that would occur preferentially in hypoxic vascular territories. Because the AE1 protein binds both deoxyhemoglobin and methemoglobin and may channel nitrite, AE1 could serve to localize catalytic NO and S-nitrosothiol generation at the erythrocyte membrane, where the relatively lipophilic NO, NO_2 and N_2O_3 could react in the vicinal lipid bilayer (Figure 5). The erythrocyte membrane is lined by an unstirred outer diffusion barrier and an inner methemoglobin rich protein matrix that might further promote such NO and NO_2 chemistry.[42-44]

This model is consistent with the *in vitro* observations of Pawloski and colleagues[45] showing that S-nitrosation of hemoglobin and AE1 occurs in the erythrocyte membrane after treatment of deoxygenated red blood cells with NO solutions (which contain significant-more than 50 μM- contaminating nitrite[37]). Further, N_2O_3 generated at the membrane could directly nitrosate the abundant intra-erythrocytic glutathione, eliminating the requirement of transnitrosation reactions with S-nitroso-hemoglobin and thus facilitating rapid export of low molecular weight S-nitrosothiol by simple diffusion across the erythrocyte membrane (Figure

5). A nitrite-hemoglobin chemistry supports a role for the red cell in oxygen-dependent NO homeostasis and provides a mechanism for the observations of multiple research groups that red blood cells and plasma "loaded" with NO, by exposure to NO in high concentration in solution or to NO gas or donors (in equilibria with high concentrations of nitrite), can export NO and induce vasodilation *in vitro* and *in vivo*.[11,27-32]

In addition to the reaction of nitrite with deoxyhemoglobin, reactions with deoxy-myoglobin, -cytoglobin and -neuroglobin or with other endothelial cell heme proteins may also be important. Such chemistry would occur between tissue nitrite and deoxy-myoglobin in vascular and skeletal muscle, thus contributing to hypoxic vasodilation and hypoxic potentiation of NO donors. The P_{50} of these globin monomers is approximately 3-5 mm Hg, placing their equilibrium deoxygenation point in the range of tissue pO_2 (0-10 mm Hg) during metabolic stress, such as exercise. Such a low oxygen tension reduces oxygen availability as substrate for NO synthesis, however, the tissue nitrite stores could then be reduced to NO and S-nitrosothiol, thus sustaining critical vasodilation.

Methods

Human subjects protocol.

The protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed consent was obtained from all volunteer subjects. Nine men and nine women, with an average age of 33 years (range 21 - 59 years), participated in the study. Volunteers had a normal hemoglobin concentration, and all were in excellent general health without risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, low-density lipoprotein cholesterol >130 mg/dL, blood pressure >145/95 mmHg, smoking within two years, cardiovascular disease, peripheral vascular disease, coagulopathy, or any other disease predisposing to vasculitis or Raynaud's phenomenon). Subjects with G6PD deficiency, known cytochrome B5 deficiency or a baseline methemoglobin level > 1% were excluded (no screened patients met these exclusion criteria). Lactating and pregnant females were excluded (one patient with positive

HCG levels was excluded). No volunteer subject was allowed to take any medication (oral contraceptive agents allowed), vitamin supplements, herbal preparations, nutriceuticals or other "alternative therapies" for at least one month prior to study and were not be allowed to take aspirin for one week prior to study.

Forearm blood flow measurements

Brachial artery and antecubital vein catheters were placed into the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump delivering normal saline at 1 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as previously reported.[46] A series of 7 blood flow measurements were averaged for each blood flow determination. A series of measurements termed Parts I and II were performed in randomized order to minimize a time effect on the forearm blood flow response during nitrite infusion.

Measurement of blood flow and forearm nitrite extraction during NO blockade and repetitive exercise

Part I: Following 20 minutes of 0.9% NaCl (saline) solution infusion at 1 mL/min into the brachial artery, arterial and venous blood samples were obtained for the assays described below and forearm blood flow measured. Exercise was performed by repetitive hand-grip at one-third of the predetermined maximum grip strength using a hand-grip dynamometer (Technical Products Co.).[8,29] Each contraction lasted for 10 seconds followed by relaxation for 5 seconds. Following 5 minutes of exercise, forearm blood flow measurements were obtained during relaxation phases of exercise, and arterial and venous samples collected. Following a 20-minute rest period with continued infusion of saline into the brachial artery, repeated baseline blood samples and forearm blood flow measurements were obtained. L-NMMA was then infused at a rate of 1 mL/min (8 μmol/min) into the brachial artery. Following 5 minutes of L-NMMA infusion, forearm blood flow was measured, and arterial and venous blood samples obtained. Forearm exercise

was then initiated in that arm during continued L-NMMA infusion. Forearm blood flow was measured and blood samples obtained after 5 minutes of exercise during continued L-NMMA infusion (Figure 1).

Part II: After a 30 minute rest period with continued infusion of saline, baseline measurements were obtained, the saline infusion was then stopped, and infusion of nitrite ($NaNO_2$ 36 μmol/ml in 0.9% saline) at 1 ml/min was started. Sodium nitrite for use in humans was obtained from Hope Pharmaceuticals (300 mg in 10 ml water) and 286 mg was diluted in 100 ml 0.9% saline by the Pharmaceutical Development Service to a final concentration of 36 μmol/ml. For the final 9 subjects studied, 0.01-0.03 mM sodium bicarbonate was added to the normal saline, so as to titrate pH to 7.0-7.4. The nitrite solution was light protected and nitrite levels and free NO gas in solution measured by reductive chemiluminescence after all experiments.[30] Only 50.5 ± 40.5 nM NO was present in nitrite solutions and was unaffected by bicarbonate buffering. There was no correlation between NO levels in nitrite solutions and blood flow effects of nitrite ($r = -0.23$; $P=0.55$). After 5 minutes of nitrite infusion, forearm blood flow measurements and blood samples were obtained. With continued nitrite infusion, exercise was performed as described previously, with forearm blood flow measurements and blood samples obtained as described above. The nitrite infusion was stopped and saline infusion re-started during the subsequent 30-minute rest period. Then nitrite infusion was re-initiated, along with L-NMMA at 8 μmol/min. Five minutes later, forearm blood flow measurements were performed and blood samples obtained followed by 5 minutes of exercise with continuation of nitrite and L-NMMA infusions. Final forearm blood flow measurements and blood samples obtained. At all time points during part II, blood samples were obtained from the contralateral arm antecubital vein for determination of methemoglobin and systemic levels of NO-modified hemoglobin (Figure 2, 3, and 4). The total dose of sodium nitrite infused in our study participants was 36 μmol/min x 15 minutes x 2 infusions = 1.08 mmol = 75 mg (MW $NaNO_2$ = 69), which is approximately one-third the dose used in humans for cyanide poisoning.

Arterial and venous pH, pO_2, and pCO_2, were measured at the bedside using the i-STAT system (i-STAT Corporation, East Windsor, NJ) and methemoglobin concentration and hemoglobin oxygen saturation measured by co-oximetry.

Measurement of red blood cell S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin.

S-nitroso-hemoglobin is unstable in the reductive red blood cell environment and rapidly decays in a temperature and redox dependent fashion, independent of oxygen tension.[30] In order to stabilize the S-nitroso-hemoglobin for measurement, the red blood cell is rapidly oxidized with ferricyanide. Before and during nitrite infusions, blood was drawn from both the brachial artery and antecubital vein and the whole blood immediately (at the bedside to eliminate processing time) lysed 1:10 in an NO-hemoglobin "stabilization solution" of PBS containing 1% NP-40 (to solubilize membranes), 8 mM NEM (to bind free thiol and prevent artefactual S-nitrosation), 0.1 mM DTPA (to chelate trace copper), and 4 mM ferricyanide and cyanide (to stabilize S-nitrosohemoglobin and prevent artefactual ex-vivo iron-nitrosylation during processing). The samples were desalted across a 9.5 mL bed volume Sephadex G25 column to eliminate nitrite and excess reagents and partially purify hemoglobin (99% hemoglobin preparation). The hemoglobin fraction was quantified by the method of Drabkin, and hemoglobin fractions reacted with and without mercuric chloride (1:5 $HgCl_2$:heme ratio- used to differentiate S-nitrosothiol which is mercury labile vs iron-nitrosyl which is mercury stable) and then in 0.1 M HCL/0.5%sulfanilamide (to eliminate residual nitrite[47]). The samples were then injected into a solution of tri-iodide (I_3^-) in-line with a chemiluminescent nitric oxide analyzer (Sievers, Model 280 NO analyzer, Boulder, CO). The mercury stable peak represents iron-nitrosyl-hemoglobin. This assay is sensitive and specific for both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin to 5 nM in whole blood (0.00005% SNO per heme).

Analysis was initially performed using red blood cell pellet, however, it was found that despite placing the sample in ice and immediately separating plasma from erythrocyte pellet, NO formed in the venous blood *ex vivo*. In order to measure the true *in vivo* levels whole blood was mixes at the bedside 1:10 in the

"NO-hemoglobin stabilization solution". Plasma S-nitroso-albumin formation was negligible during nitrite infusion, so this bedside whole blood assay was used to limit processing time and thus more accurately characterize the *in vivo* chemistry. In a series of validation experiments it was found that both S-nitroso-hemoglobin and iron-nitrosyl-hemoglobin were stable in the "NO-hemoglobin stabilization solution" for 20 minutes at room temperature with no artifactual formation or decay of NO-modified species (n=6; data not shown).

Electron Paramagnetic Resonance Spectroscopy of Whole Blood was carried out at 137 K with a Bruker 4111 VT controller and ER-200 D ESR spectrometer set at 9.43 GHz, 10 mW, 5 G modulation, 0.1 s time constant and 100 s scans over 600 G. Each curve represents the average of ten 100 s scans. Arterial blood spectra were subtracted from venous blood spectra, demonstrating an increase in iron-nitrosyl-hemoglobin from artery-to-vein. Difference spectra from three patients are shown.

Statistical analysis.

An a priori sample size calculation determined that 18 subjects would be necessary for the study to detect a 25% improvement in forearm blood flow during nitrite infusion when forearm NO synthesis had been inhibited by L-NMMA compared with normal saline infusion control values (alpha=0.05, power=0.80). Two-sided P values were calculated by paired t-test for the pair-wise comparisons between baseline and L-NMMA infusion values, between baseline and exercise values, and between nitrite and saline control values at comparable time-points of the study. Repeated measures ANOVA was performed for artery-to-vein gradients of NO species during basal, L-NMMA infusion, and exercise conditions. Measurements shown are mean ± SEM.

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All publications, patents and patent applications cited herein are herein incorporated by reference.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

The following statements of the invention are intended to characterize possible elements of the invention according to the foregoing description given in the specification. Because this application is a provisional application, these statements may become changed upon preparation and filing of a nonprovisional application. Such changes are not intended to affect the scope of equivalents according to the claims issuing from the nonprovisional application, if such changes occur. According to 35 U.S.C. § 111(b), claims are not required for a provisional application. Consequently, the statements of the invention cannot be interpreted to be claims pursuant to 35 U.S.C. § 112.

Formal Claim

1. A method for decreasing a patient's blood pressure, comprising administering to the patient sodium nitrite at 36 μmoles per minute into the forearm brachial artery.

Statements of the Invention

1. A method for decreasing a patient's blood pressure, comprising administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

2. A method for treatment of a patient with a cardiovascular malcondition, comprising administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

3. A method for treatment of a patient with a cardiovascular malcondition, comprising administering to the patient an effective amount of a pharmaceutically-acceptable compound to modulate the nitrite-deoxyhemoglobin-nitric oxide system.

4. A method to treat or prevent the deleterious ancillary effects of increased blood pressure in a patient, comprising administering to the patient an effective amount of a pharmaceutically-acceptable salt of nitrite.

5. The method of any of statements 1-4, wherein the administration is parenteral, peritoneal, oral, bucal, rectal, *ex vivo*, or intraocular.

6. The method of any of statements 1-5, wherein the administration is intravenous, intraarterial, subcutaneous or intramuscular.

7. A method of diagnosis of a malcondition in a patient, comprising determining the amount of nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide in a physiological sample from a patient and comparing the level of the nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide to the normal range of nitrite, deoxyhemoglobin, deoxymyoglobin, deoxycytoglobin, deoxyneoroglobin, and/or nitric oxide in the sample, wherein a difference in the amount of indicative of the presence and/or predictive of the development of the malcondition.

ABSTRACT

It has been discovered that nitrite is converted to nitric oxide *in vivo*, and that the nitric oxide produced thereby is an effective vasodilator. This discovery provides useful treatments to regulate a patient's blood pressure, for example, by the administration of nitride salts.

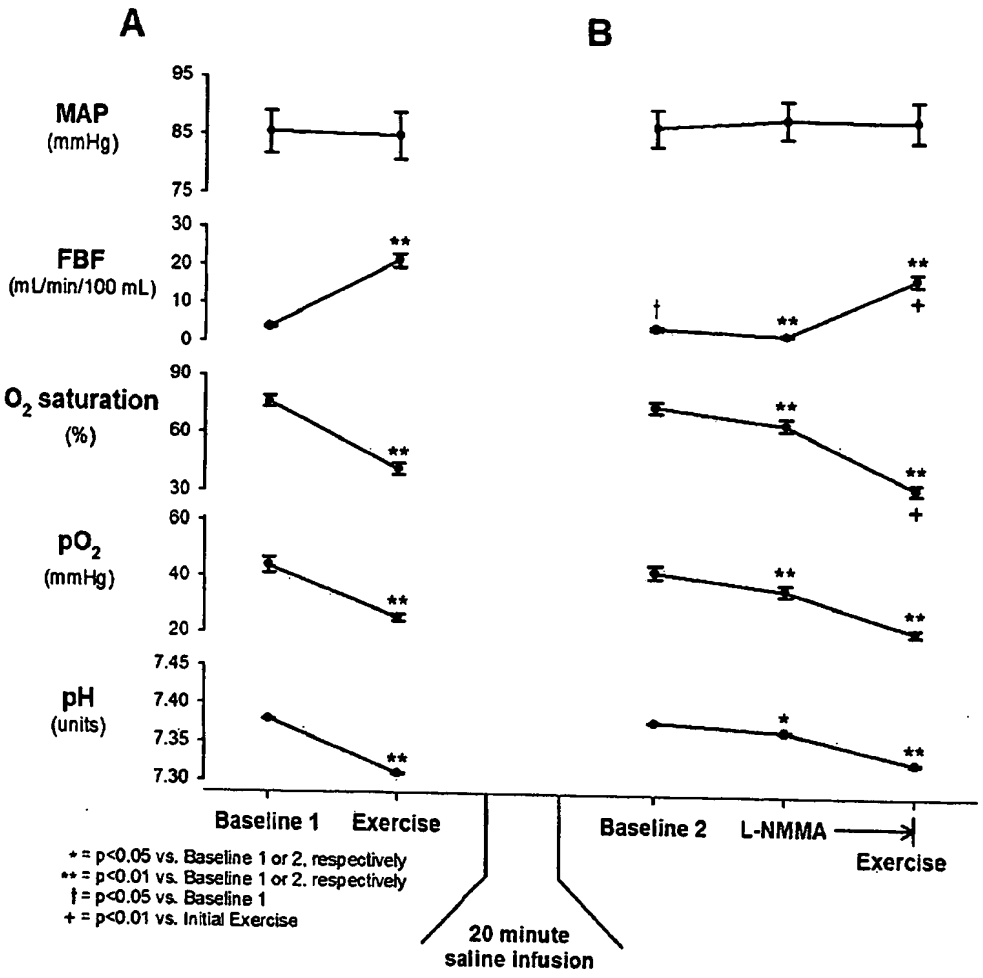

A
B
MAP
(mmHg)
95
85
75
FBF
(mL/min/100 mL)
30
20
10
0
O_2 saturation
(%)
90
60
30
pO_2
(mmHg)
60
40
20
pH
(units)
7.45
7.40
7.35
7.30
Baseline 1
Exercise
Baseline 2
L-NMMA
Exercise
20 minute
saline infusion
* = p<0.05 vs. Baseline 1 or 2, respectively
** = p<0.01 vs. Baseline 1 or 2, respectively
† = p<0.05 vs. Baseline 1
+ = p<0.01 vs. Initial Exercise

Fig. 1

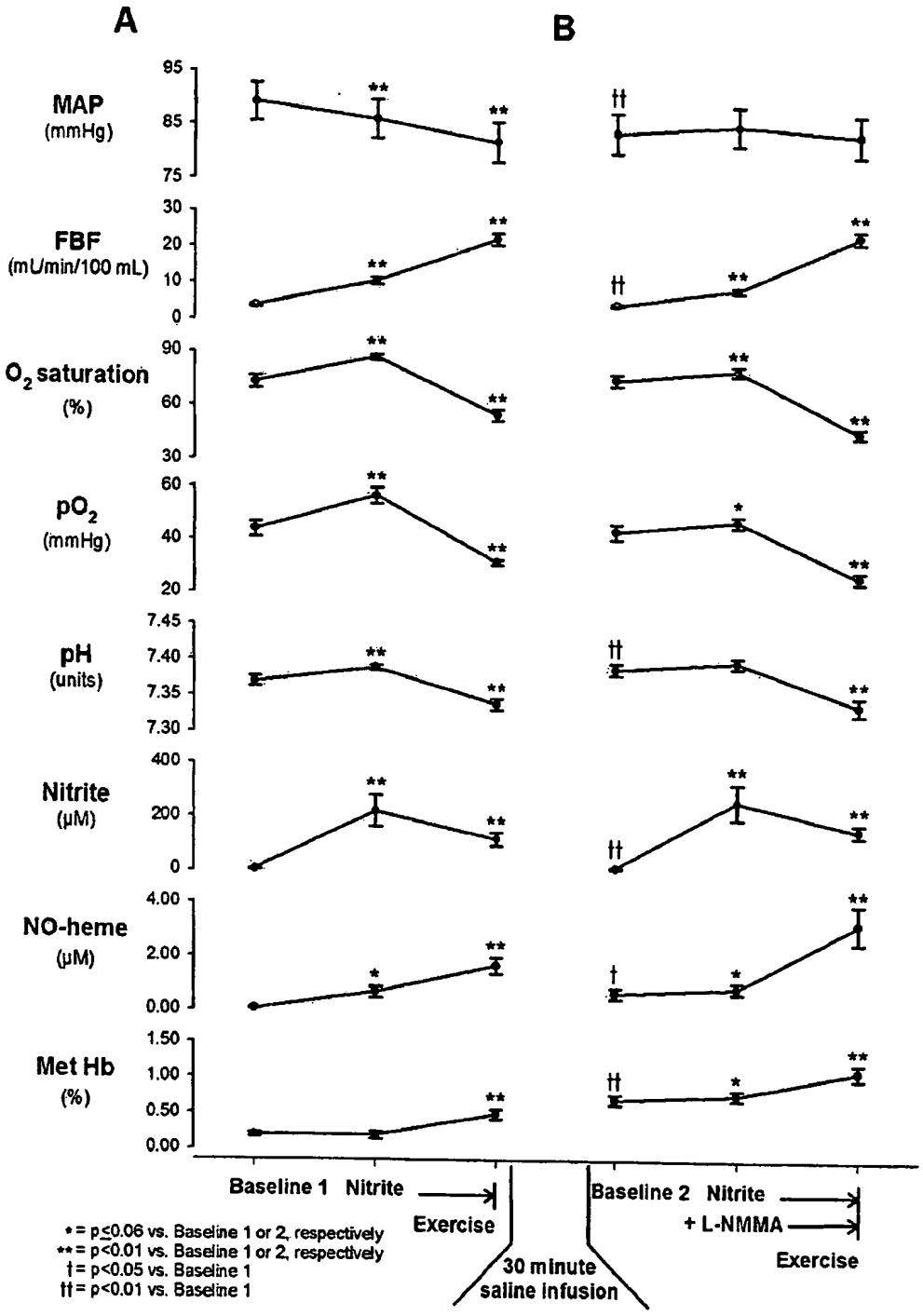
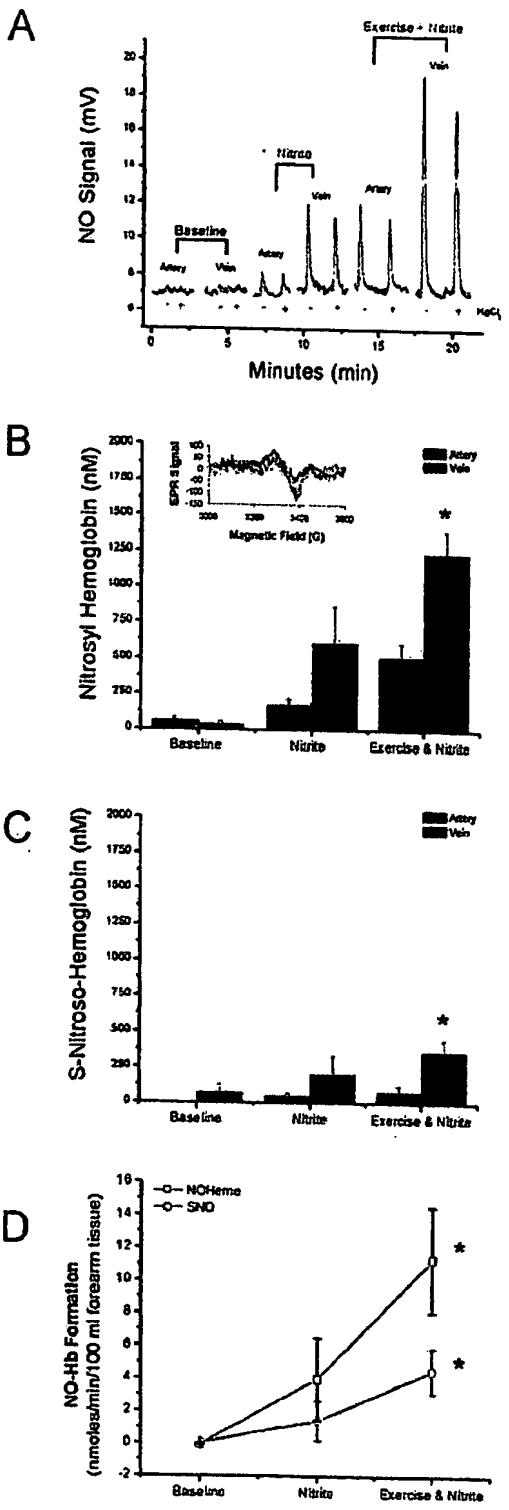
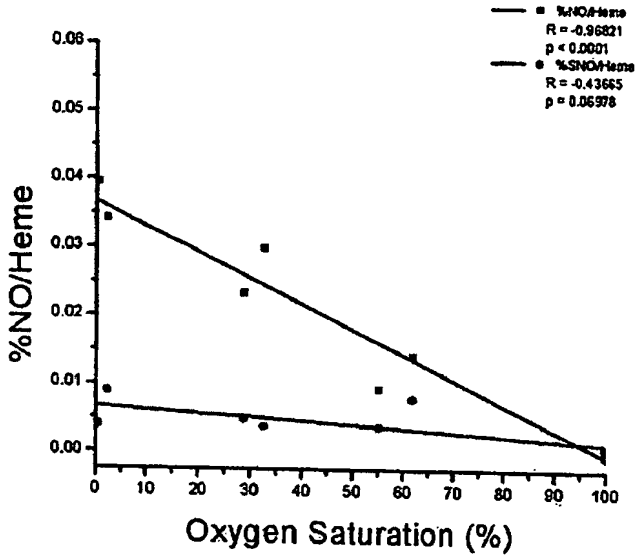

A
B
MAP
(mmHg)
95
85
75
FBF
(mL/min/100 mL)
30
20
10
0
O2 saturation
(%)
90
60
30
pO2
(mmHg)
60
40
20
pH
(units)
7.45
7.40
7.35
7.30
Nitrite
(μM)
400
200
0
NO-heme
(μM)
4.00
2.00
0.00
Met Hb
(%)
1.50
1.00
0.50
0.00
Baseline 1
Nitrite
Exercise
30 minute
saline infusion
Baseline 2
Nitrite
+ L-NMMA
Exercise
* = p≤0.06 vs. Baseline 1 or 2, respectively
** = p<0.01 vs. Baseline 1 or 2, respectively
† = p<0.05 vs. Baseline 1
†† = p<0.01 vs. Baseline 1

FIG. 2


A
NO Signal (mV)
Minutes (min)
Baseline
Nitrite
Exercise + Nitrite
Artery
Vein
B
Nitrosyl Hemoglobin (nM)
Baseline
Nitrite
Exercise & Nitrite
C
S-Nitroso-Hemoglobin (nM)
Baseline
Nitrite
Exercise & Nitrite
D
NO-Hb Formation
(nmoles/min/100 ml forearm tissue)
NOHeme
SNO
Baseline
Nitrite
Exercise & Nitrite

F16. 3

A


%NO/Heme
R = -0.96821
p < 0.0001
%SNO/Heme
R = -0.43665
p = 0.06978
%NO/Heme
Oxygen Saturation (%)

B

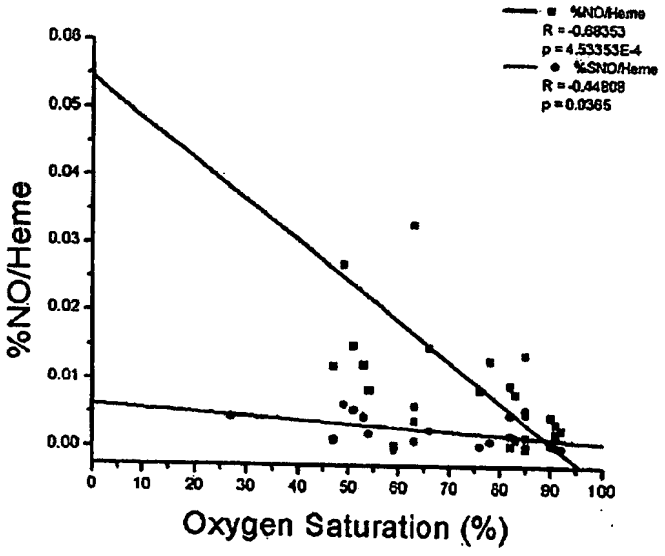

%NO/Heme
R = -0.68353
p = 4.53353E-4
%SNO/Heme
R = -0.44808
p = 0.0365
%NO/Heme
Oxygen Saturation (%)

Fig. 4

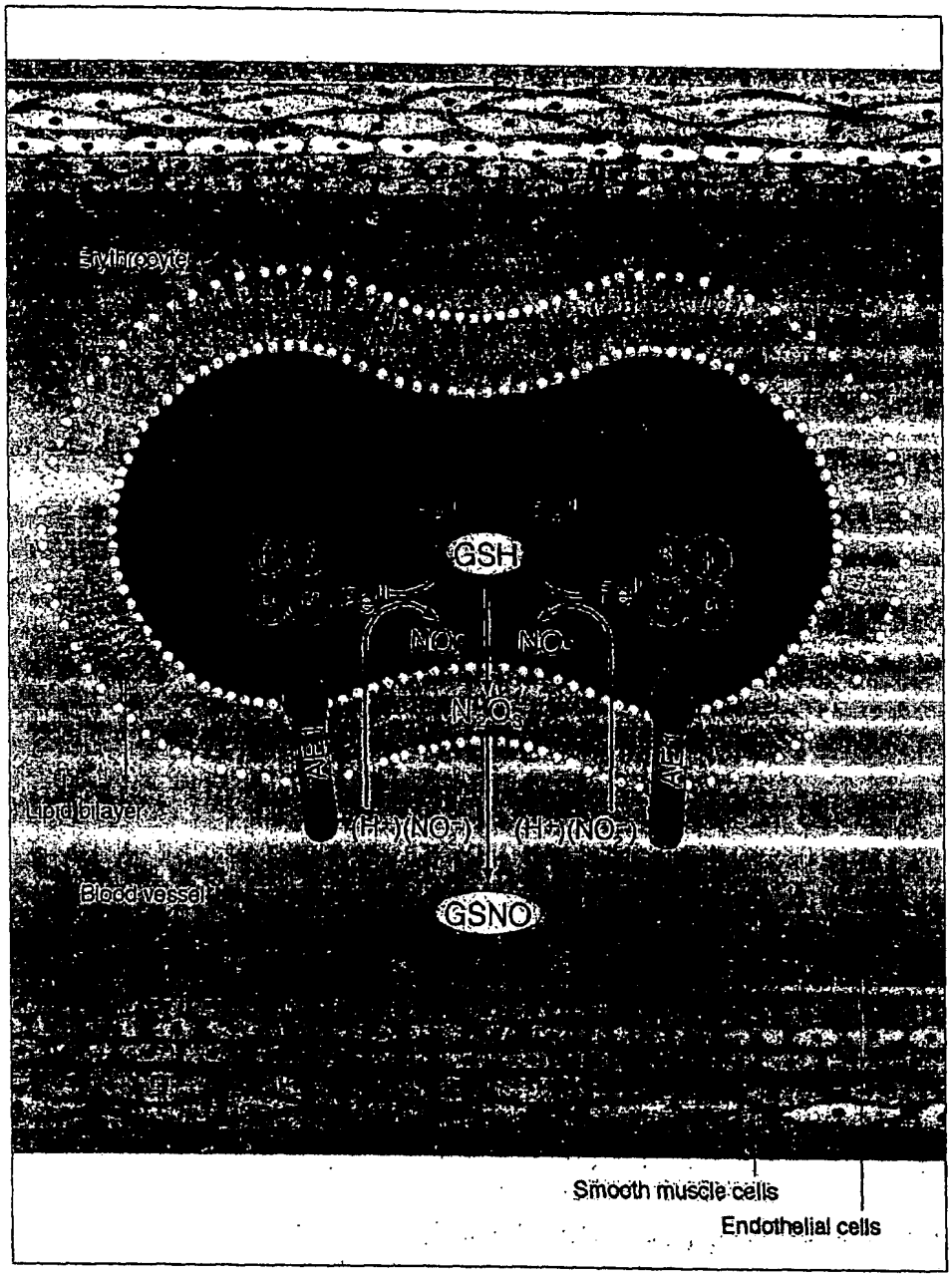

Erythrocyte
GSH
NO
NO
N_2O_3
Lipid bilayer
$(H^+)(NO_2^-)$
$(H^+)(NO_2^-)$
Blood vessel
GSNO
Smooth muscle cells
Endothelial cells

Fig. 5

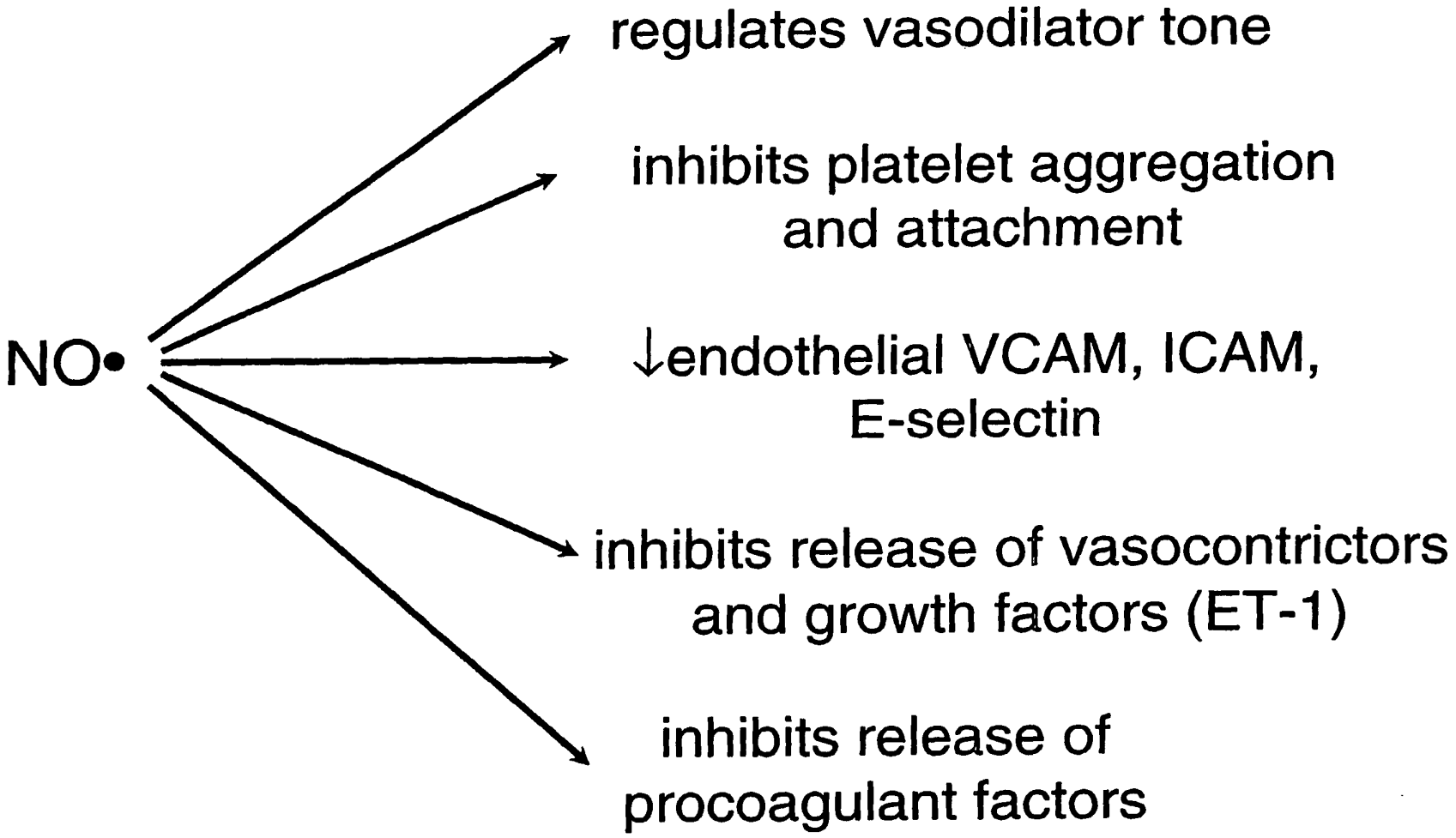

NO•
regulates vasodilator tone
inhibits platelet aggregation
and attachment
↓endothelial VCAM, ICAM,
E-selectin
inhibits release of vasocontrictors
and growth factors (ET-1)
inhibits release of
procoagulant factors

Fig. 6

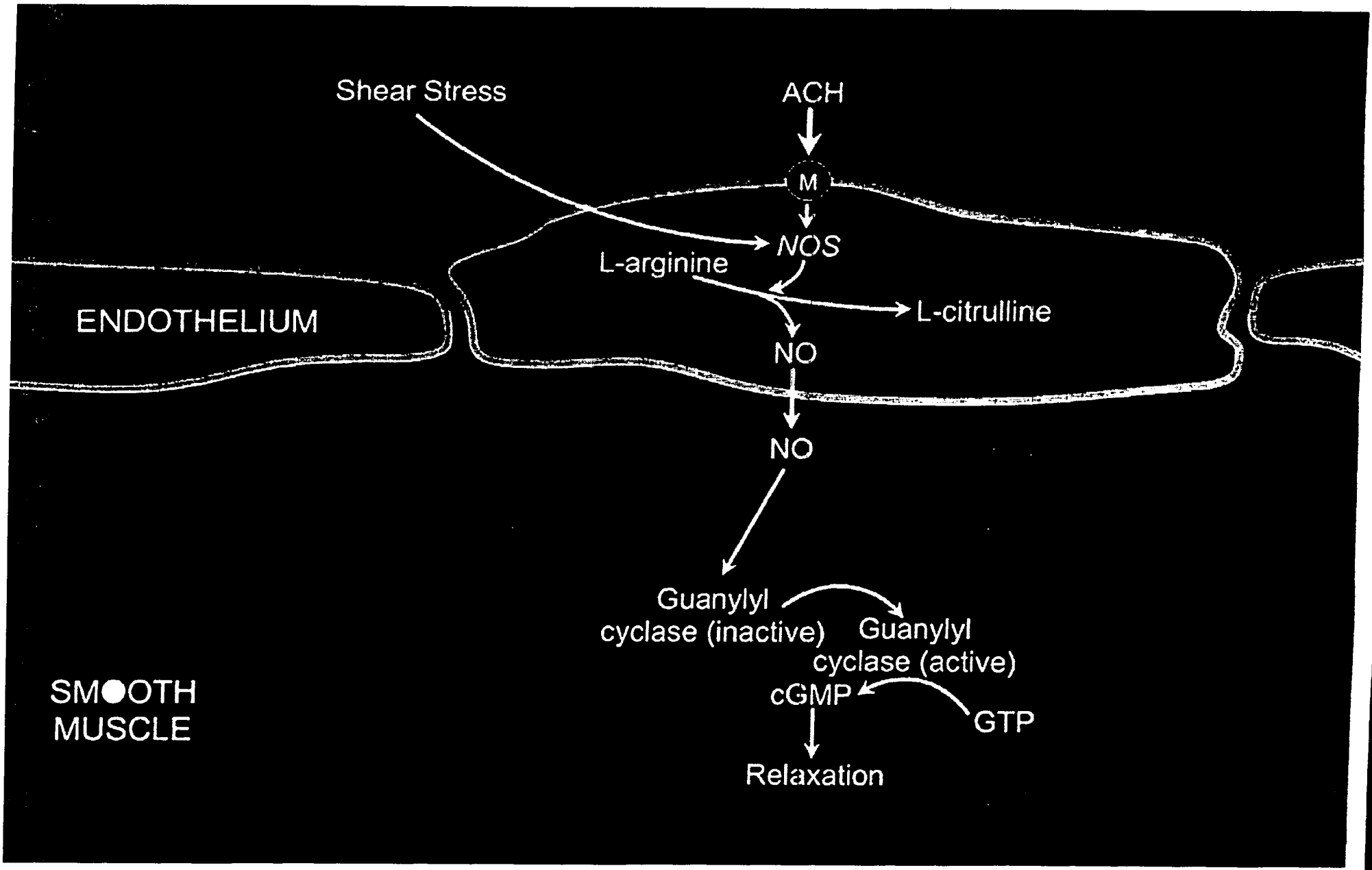

Shear Stress
ACH
M
NOS
L-arginine
L-citrulline
ENDOTHELIUM
NO
NO
Guanylyl cyclase (inactive)
Guanylyl cyclase (active)
cGMP
GTP
SMOOTH MUSCLE
Relaxation

Fig. 7

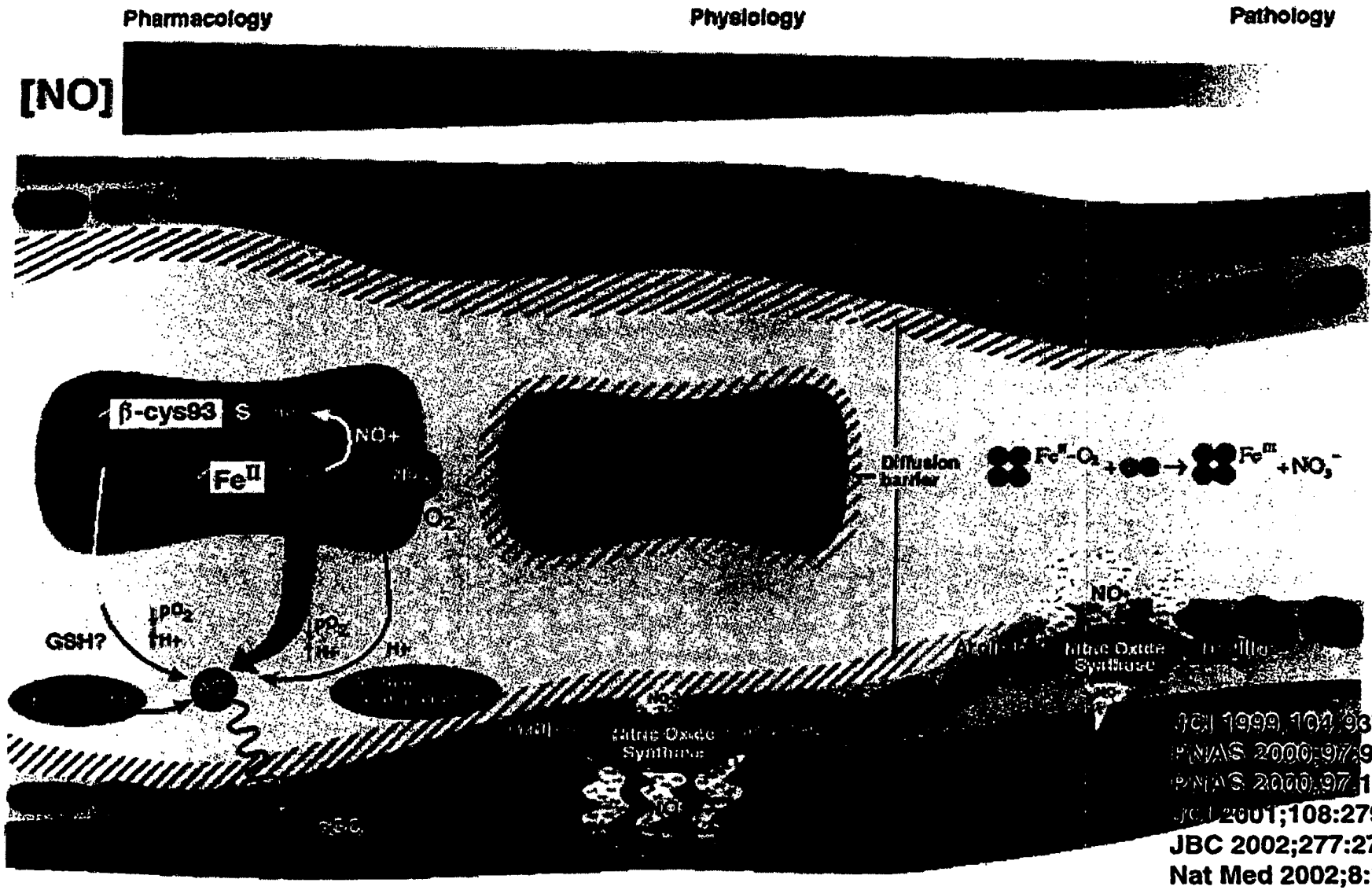

Pharmacology
Physiology
Pathology
[NO]
β-cys93
S
NO+
Fe II
O2
GSH?
Diffusion barrier
Fe II -O2 + → Fe III + NO3 -
NO
Nitric Oxide Synthase
Nitric Oxide Synthase
JCI 1999;104:937
PNAS 2000;97:9943
PNAS 2000;97:11482
JCI 2001;108:279
JBC 2002;277:27818
Nat Med 2002;8:1383
NEJM 2003;348:1483

Fig. 8

Protocol 00-H-0031: Vascular Effects of Endothelium-Derived Versus Hemoglobin-Transported Nitric Oxide in Healthy Subjects

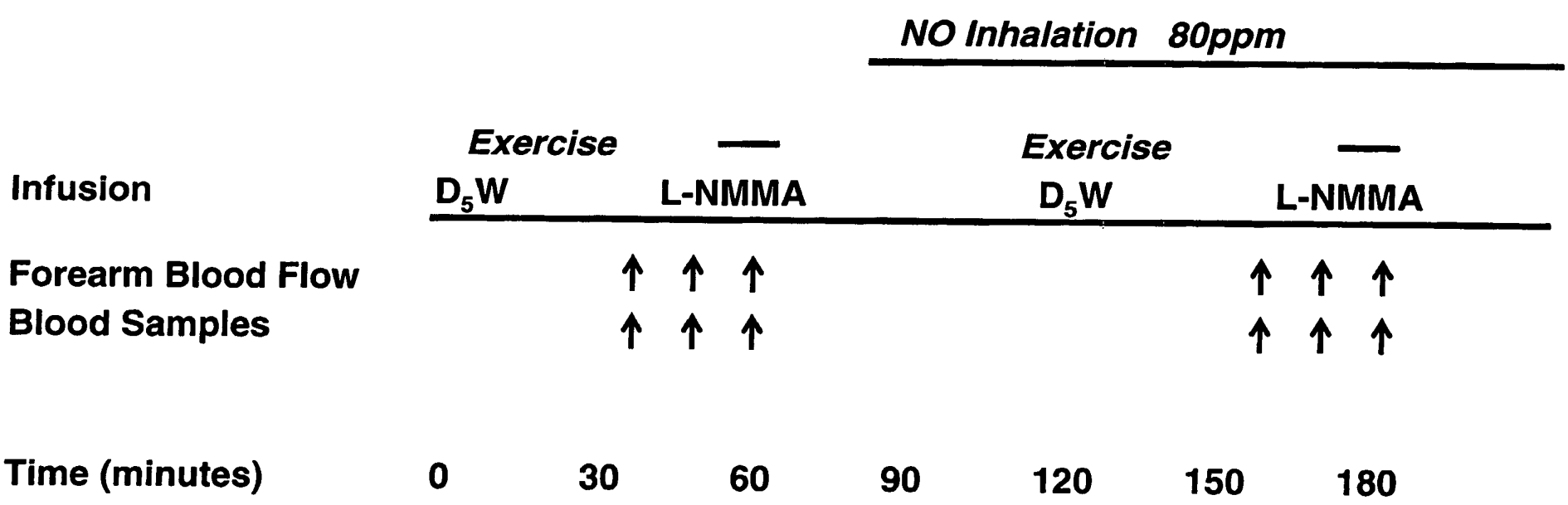


Fig. 9

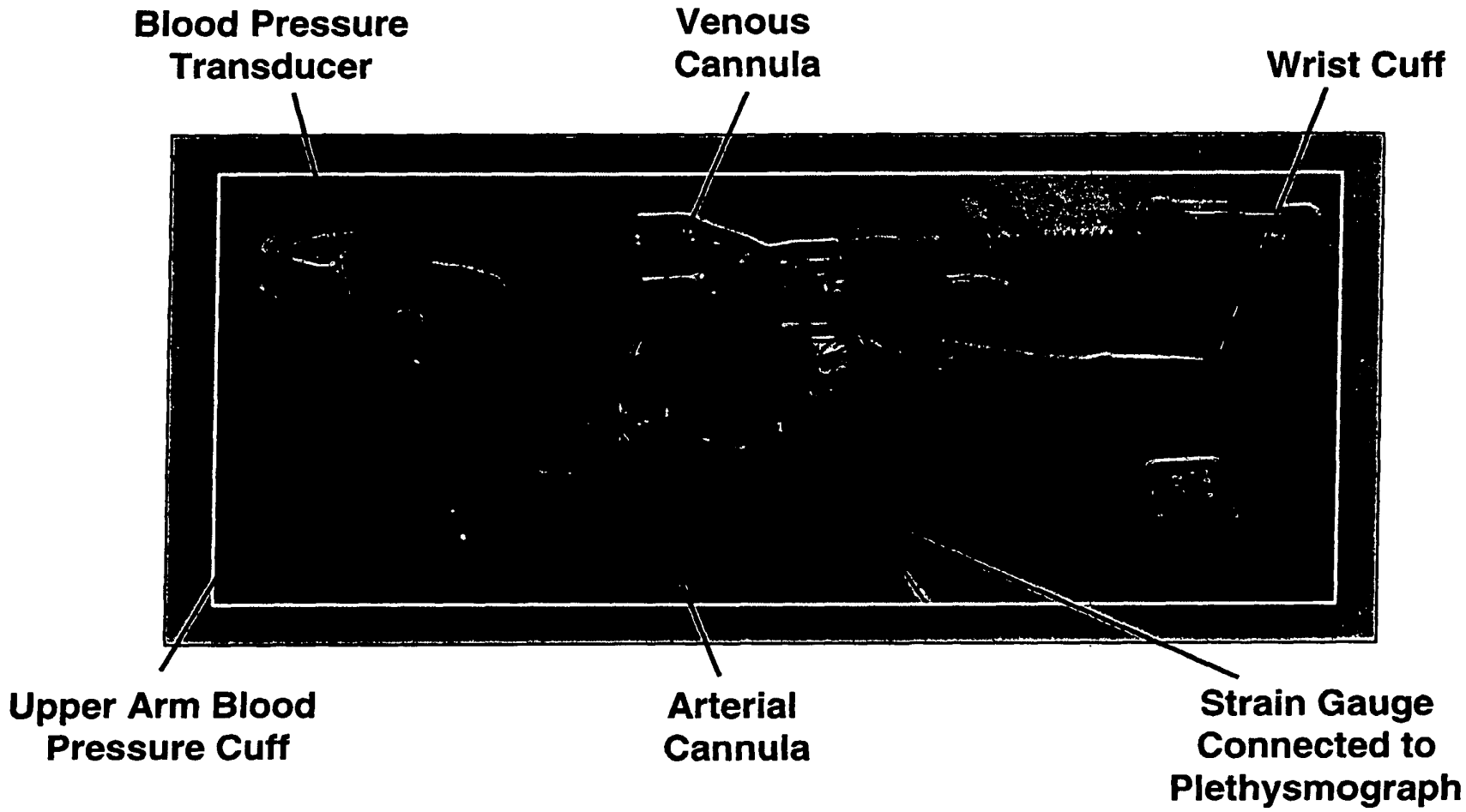

Blood Pressure Transducer
Venous Cannula
Wrist Cuff
Upper Arm Blood Pressure Cuff
Arterial Cannula
Strain Gauge Connected to Plethysmograph

FIG. 10

Nitric Oxide Breathing Restores Forearm Vasodilator Tone During Inhibition of Regional NO Synthesis

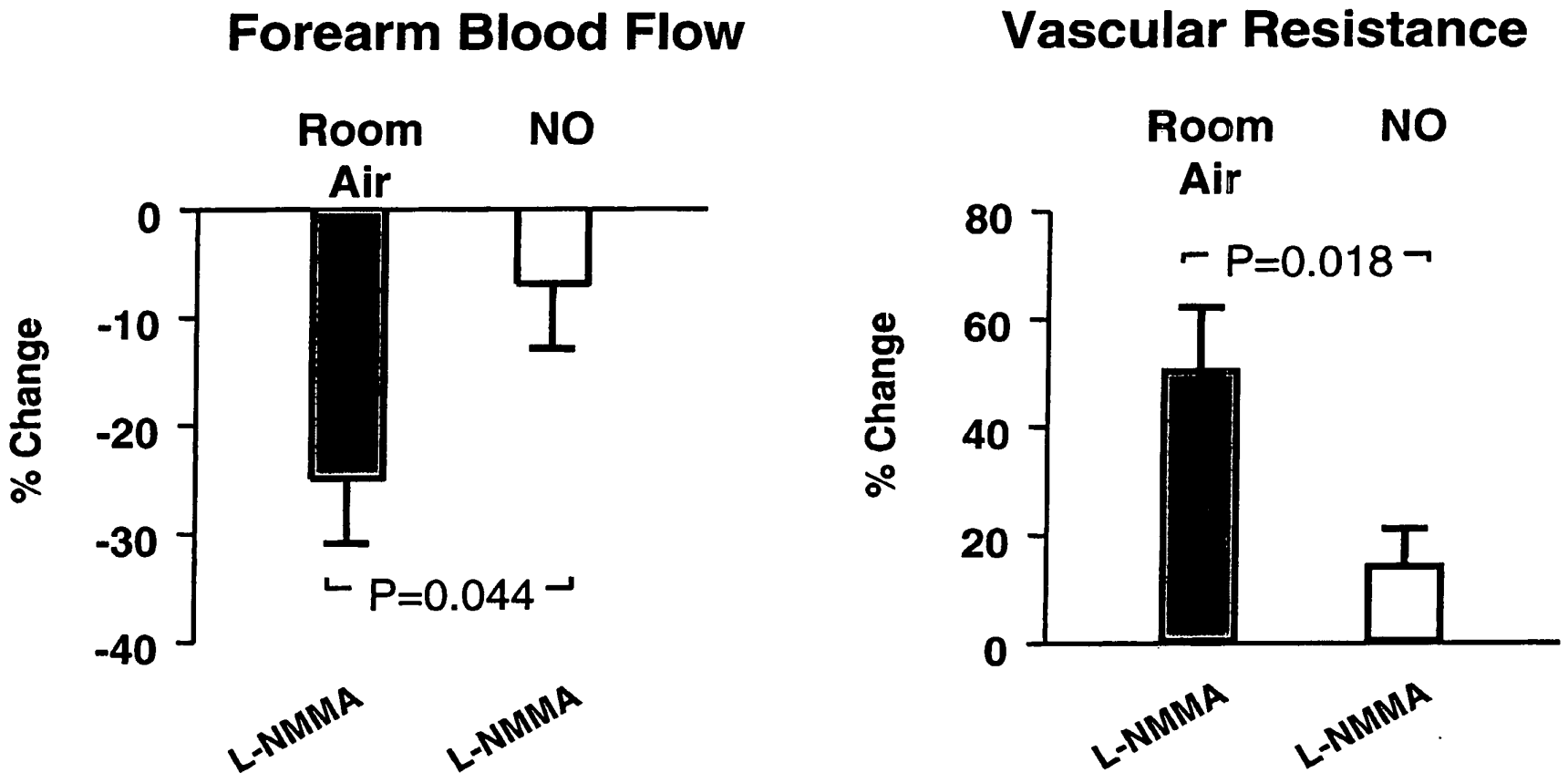


Fig. 11

Inhaled NO is Vaso-active

Nitric oxide inhalation restores blood flow during regional NOS inhibition

Results in increased tissue hemoglobin-oxygen saturation and venous pH

How is NO delivered to the peripheral vascular bed?

Fig. 12

Cannon et al. JCI 2001;108:279-87

NO Reactions with Oxygen, Thiols, and Heme

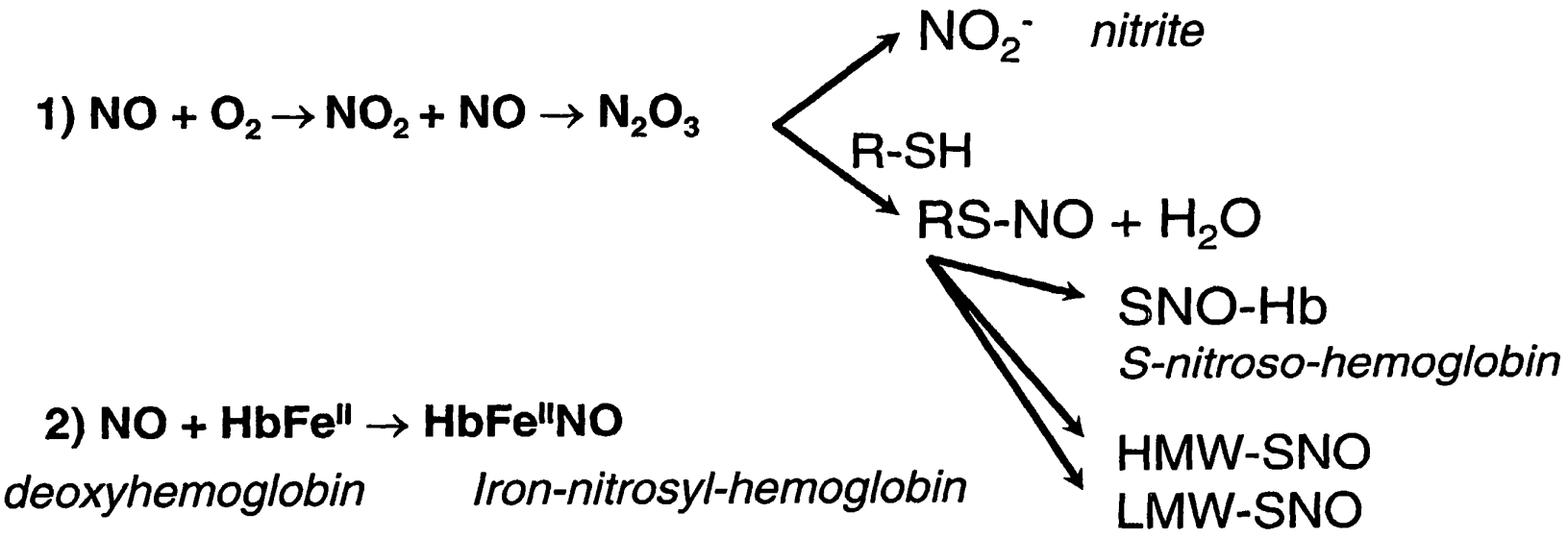
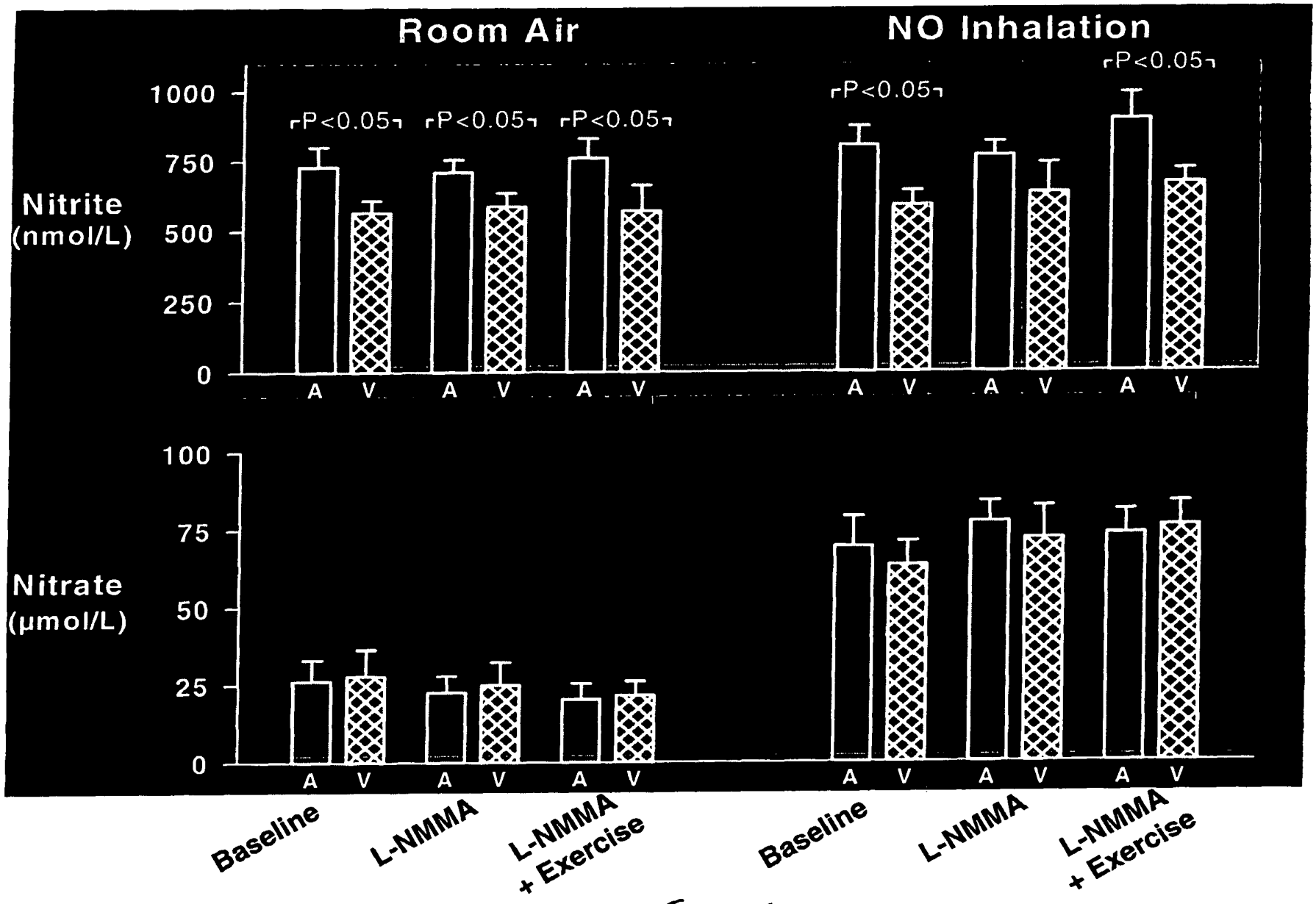


Fig. 13


Room Air
NO Inhalation
Nitrite (nmol/L)
1000
750
500
250
0
P<0.05
P<0.05
P<0.05
P<0.05
P<0.05
A V A V A V A V A V A V
Nitrate (μmol/L)
100
75
50
25
0
A V A V A V A V A V A V
Baseline
L-NMMA
L-NMMA + Exercise
Baseline
L-NMMA
L-NMMA + Exercise

F16.14

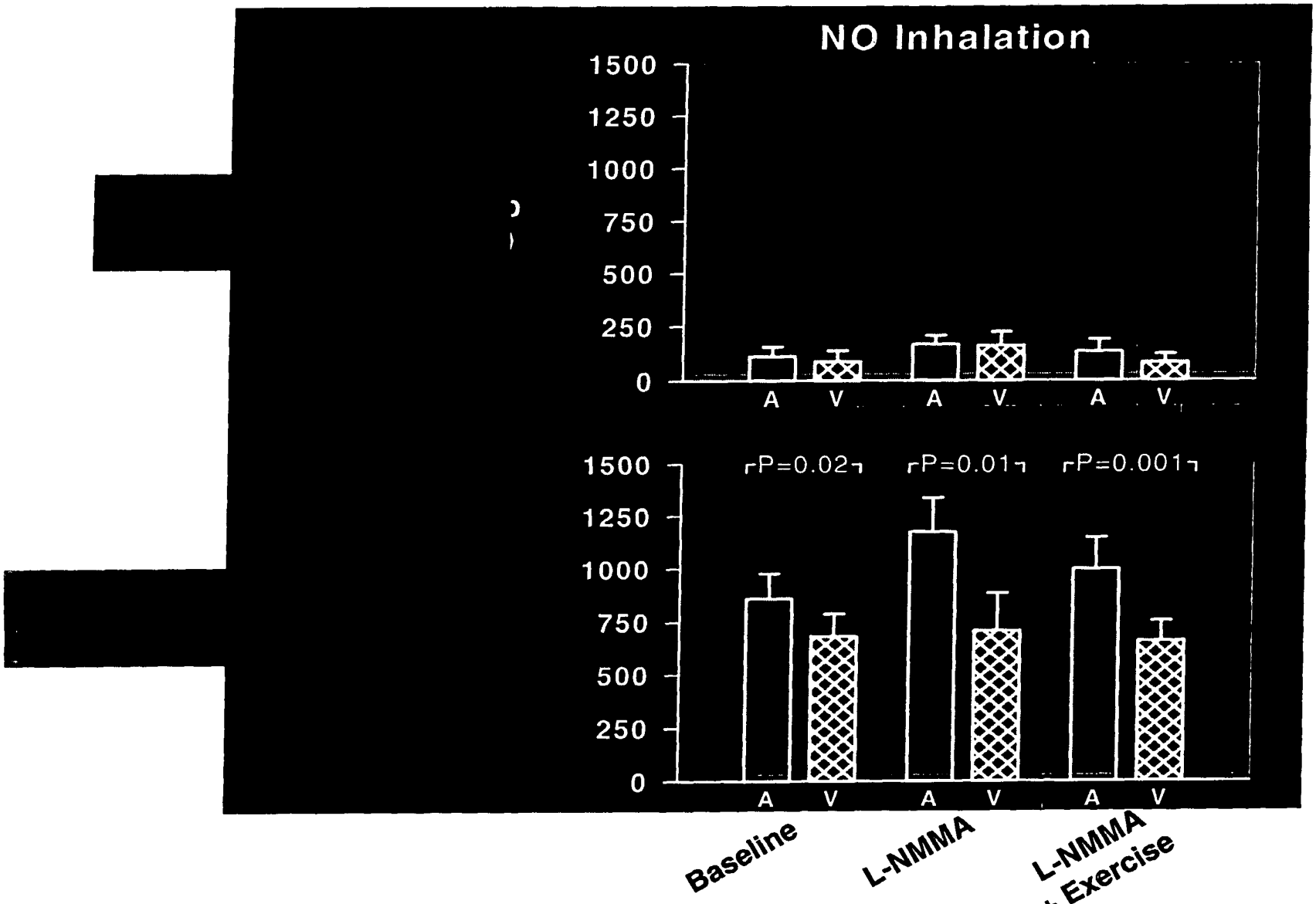
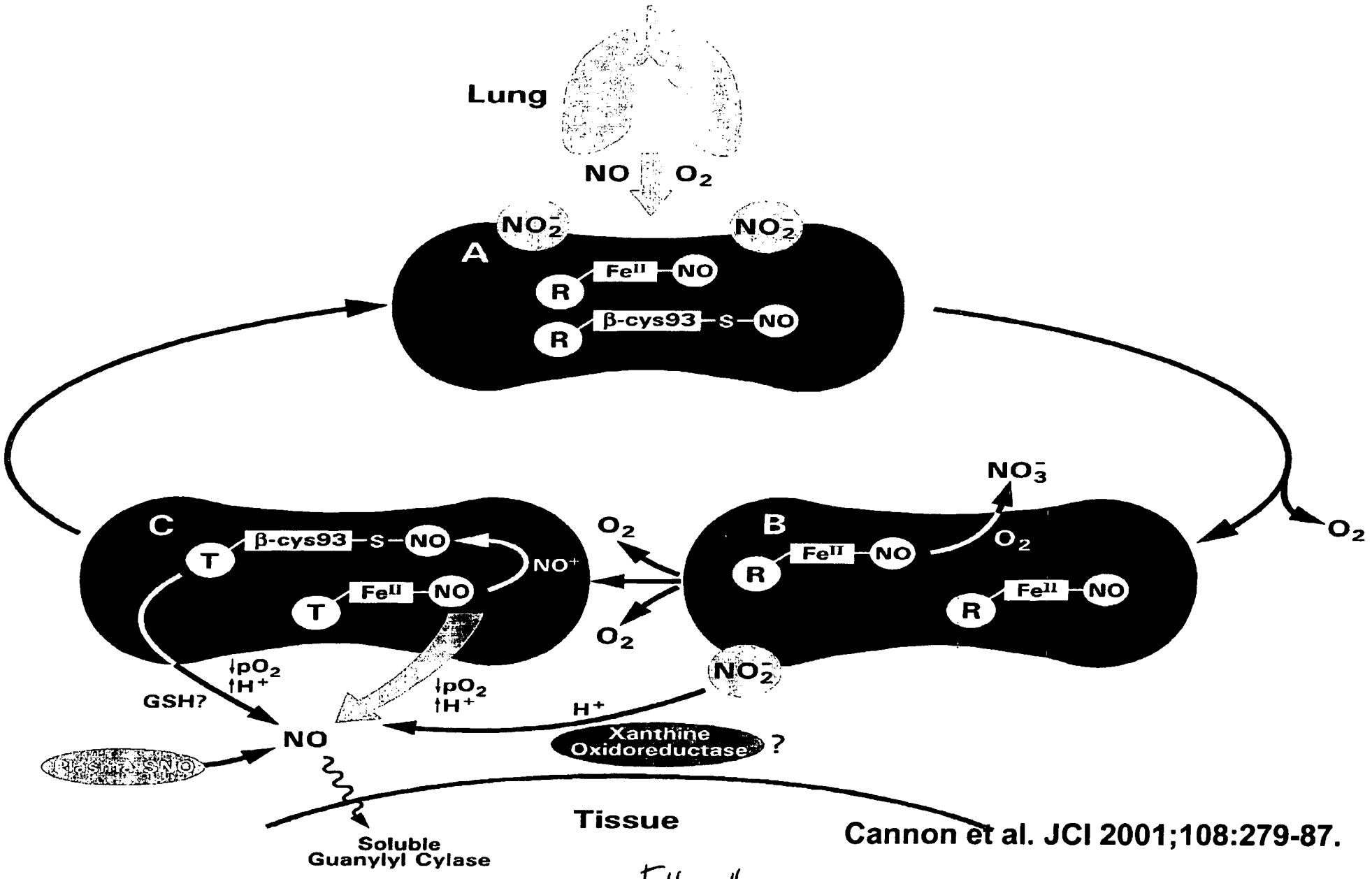

NO Inhalation
1500
1250
1000
750
500
250
0
A V A V A V
1500
1250
1000
750
500
250
0
rP=0.02¬
rP=0.01¬
rP=0.001¬
A V A V A V
Baseline
L-NMMA
L-NMMA
+ Exercise

Fig. 15



Cannon et al. JCI 2001;108:279-87.

Fig. 16

Is Nitrite a Vasodilator?

Vasodilates aortic rings at high concentration

Vasodilates lung perfusions models at concentrations of 100 μM under hypoxia

NO formation from nitrite by action of xanthine oxidase and dysproportionation require very low oxygen and pH

Levels in human blood are only 15-500 nM

Rassaf et al. PNAS: Nitrite lacks intrinsic vasodilator action in humans

Fig. 17

Protocol 00-H-0031: Determination of Nitrite as a Source of Bioactive Nitric Oxide in Human Subjects

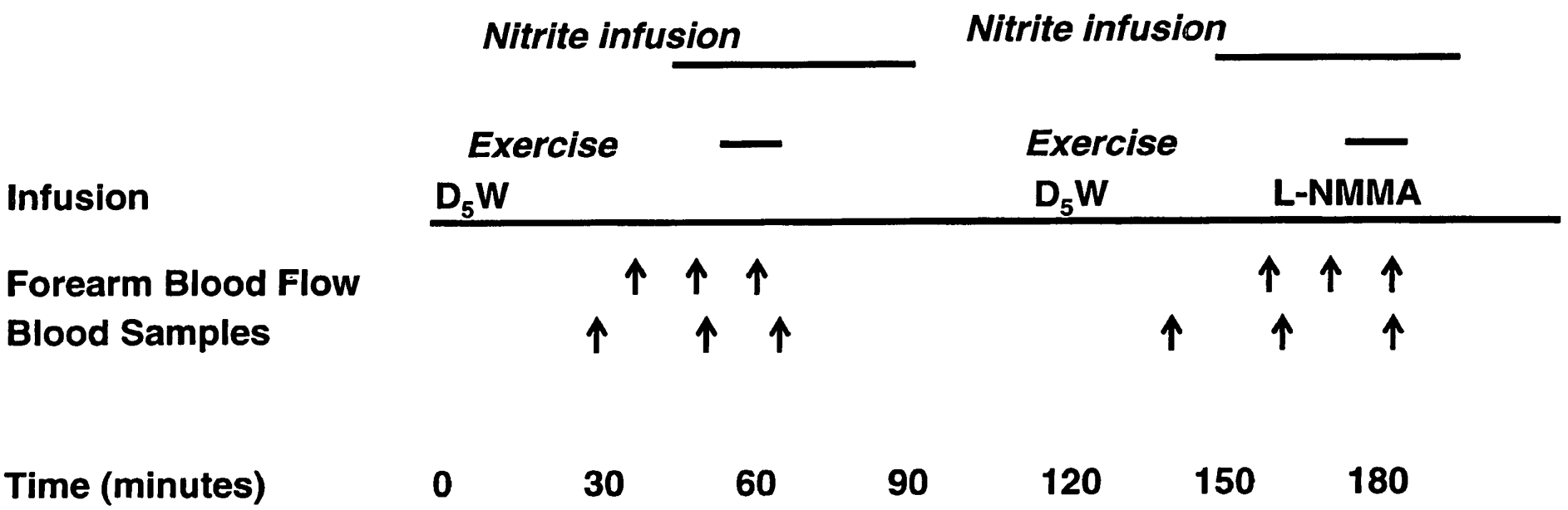


FIG. 18

Vaso-activity of Nitrite Infusion

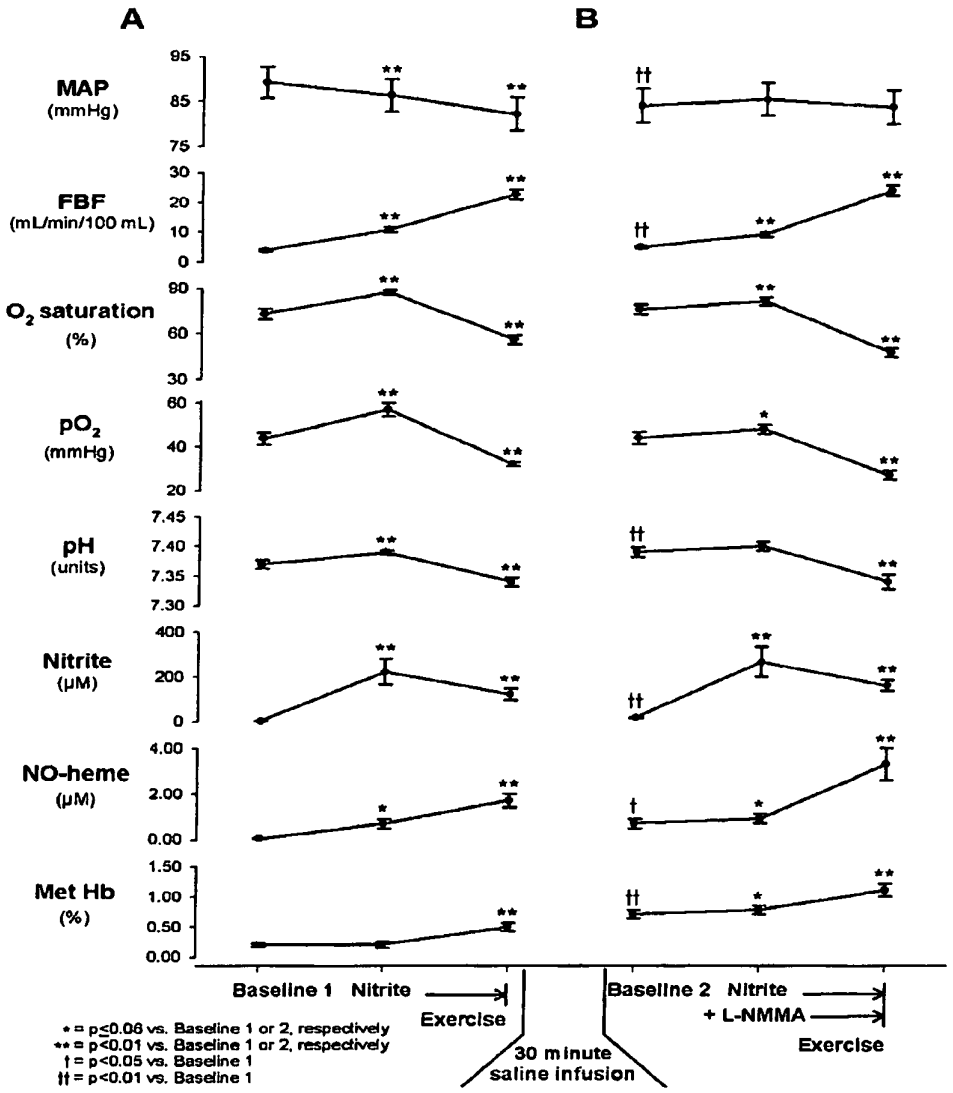
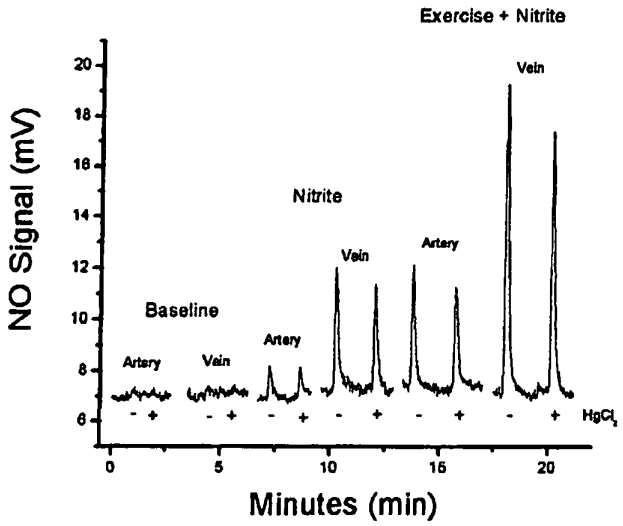
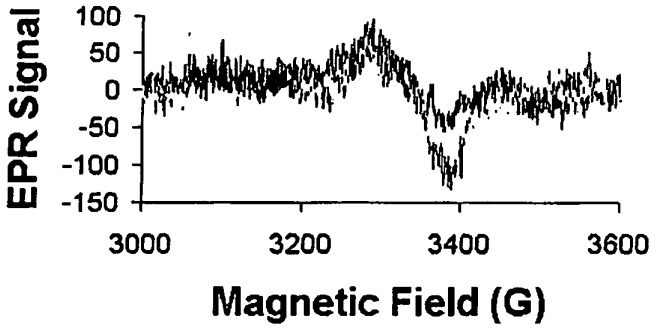
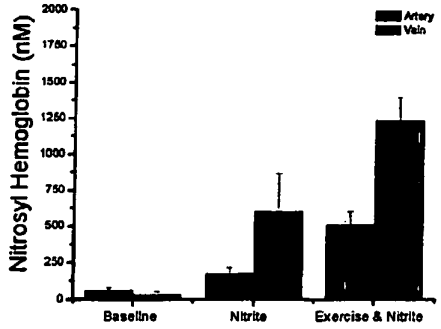


FIG. 19

Formation of NO-Hb Adducts During Nitrite Infusion







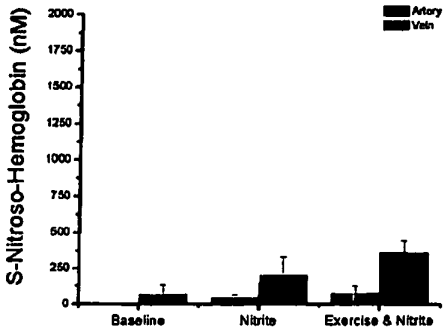


Fig. 20

NO-Hb Adducts Form from Reaction of Nitrite with Deoxy-Hemoglobin

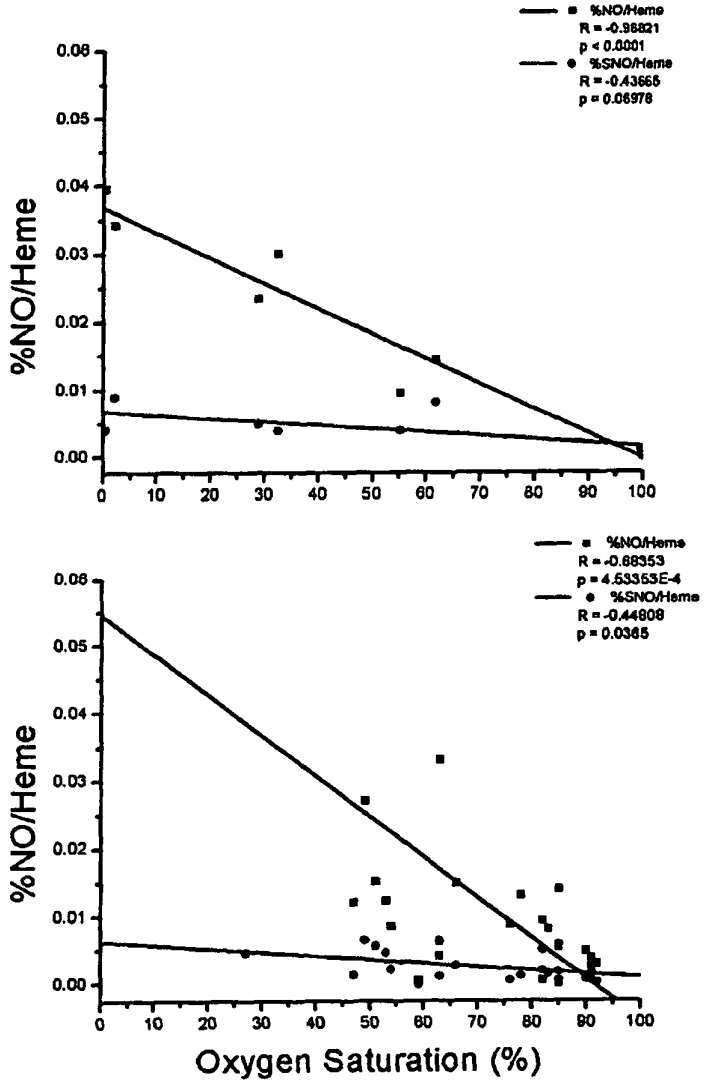


FIG. 21

Nitrite-Hemoglobin Chemistry

Equation series 1

NO_2^- (nitrite) + $HbFe^{II}$ (deoxyhemoglobin) + $H^+ \rightarrow$ $HbFe^{III}$ (methemoglobin) + $NO + OH^-$

$NO + HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}NO$ (iron-nitrosyl-hemoglobin)

Equation series 2

NO_2^- (nitrite) + $HbFe^{III} \rightarrow HbFe^{III}ONO^- \rightarrow HbFe^{II}ONO$ (Fe^{II}-nitrogen dioxide intermediate)

NO (from Equation series 1) + $HbFe^{II}ONO \rightarrow HbFe^{II}ONONO \rightarrow HbFe^{II} + N_2O_3$

N_2O_3 + R-SH $\rightarrow$ R-S-NO

FIG. 22

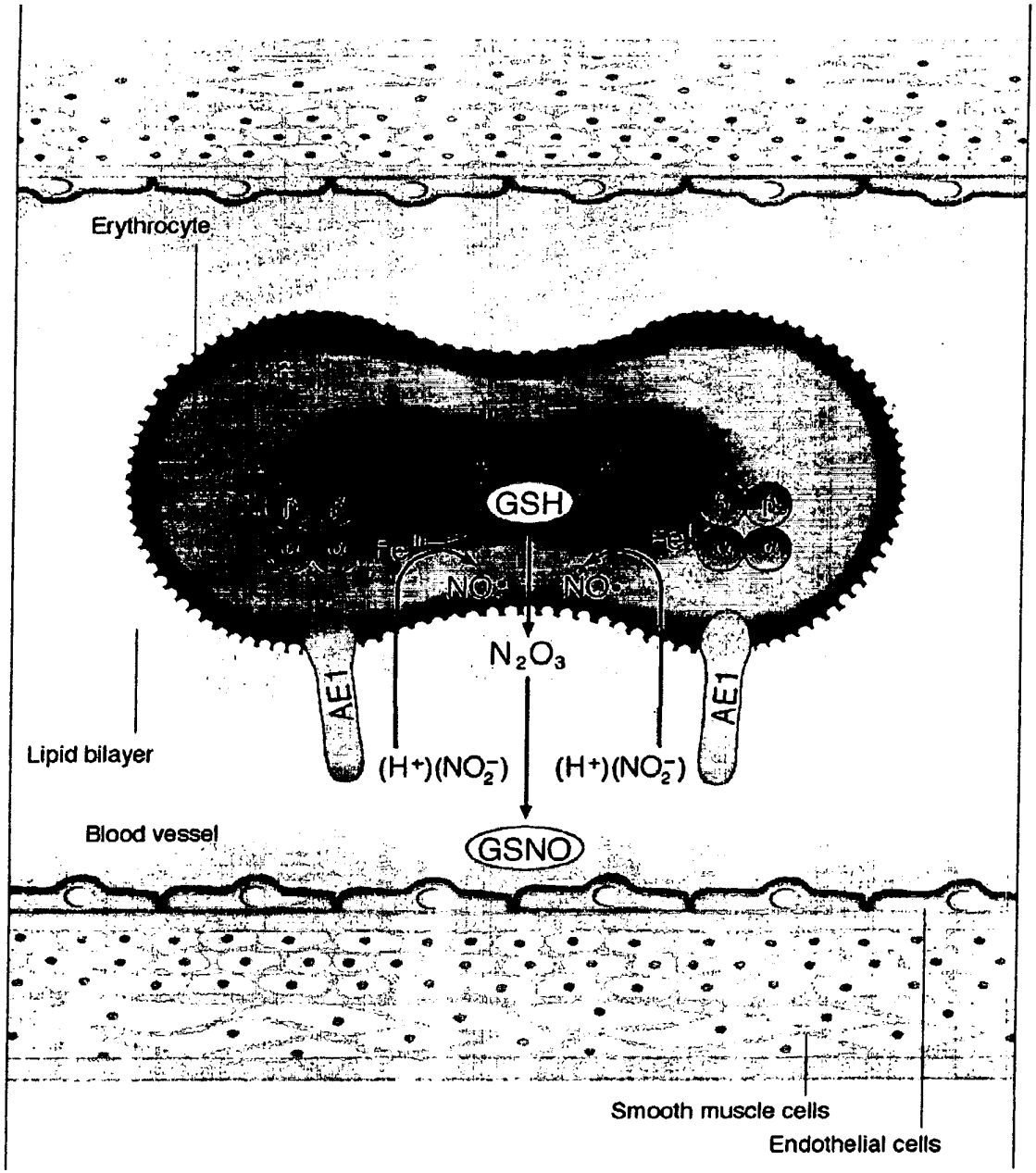
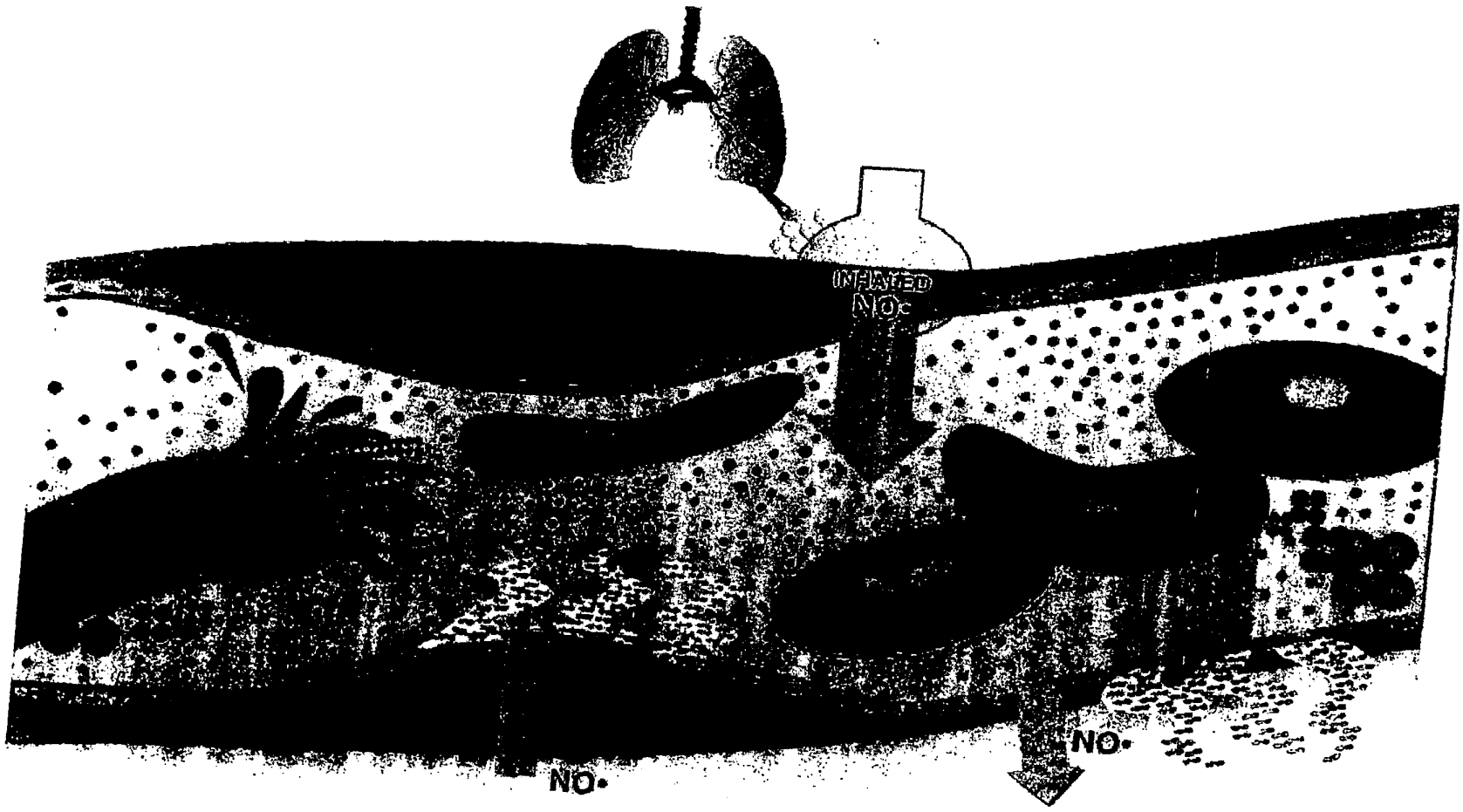

Erythrocyte
GSH
Fe II
NO
NO
N_2O_3
AE1
AE1
Lipid bilayer
$(H^+)(NO_2^-)$
$(H^+)(NO_2^-)$
Blood vessel
GSNO
Smooth muscle cells
Endothelial cells

Fig. 23



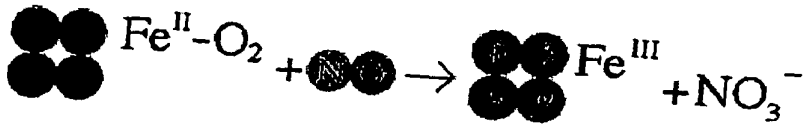


Fig. 24

Nature Med 2002; 8:1383

Relaxation Response to Nitrite at High Oxygen Tension And Low Oxygen Tension (11 mm Hg)

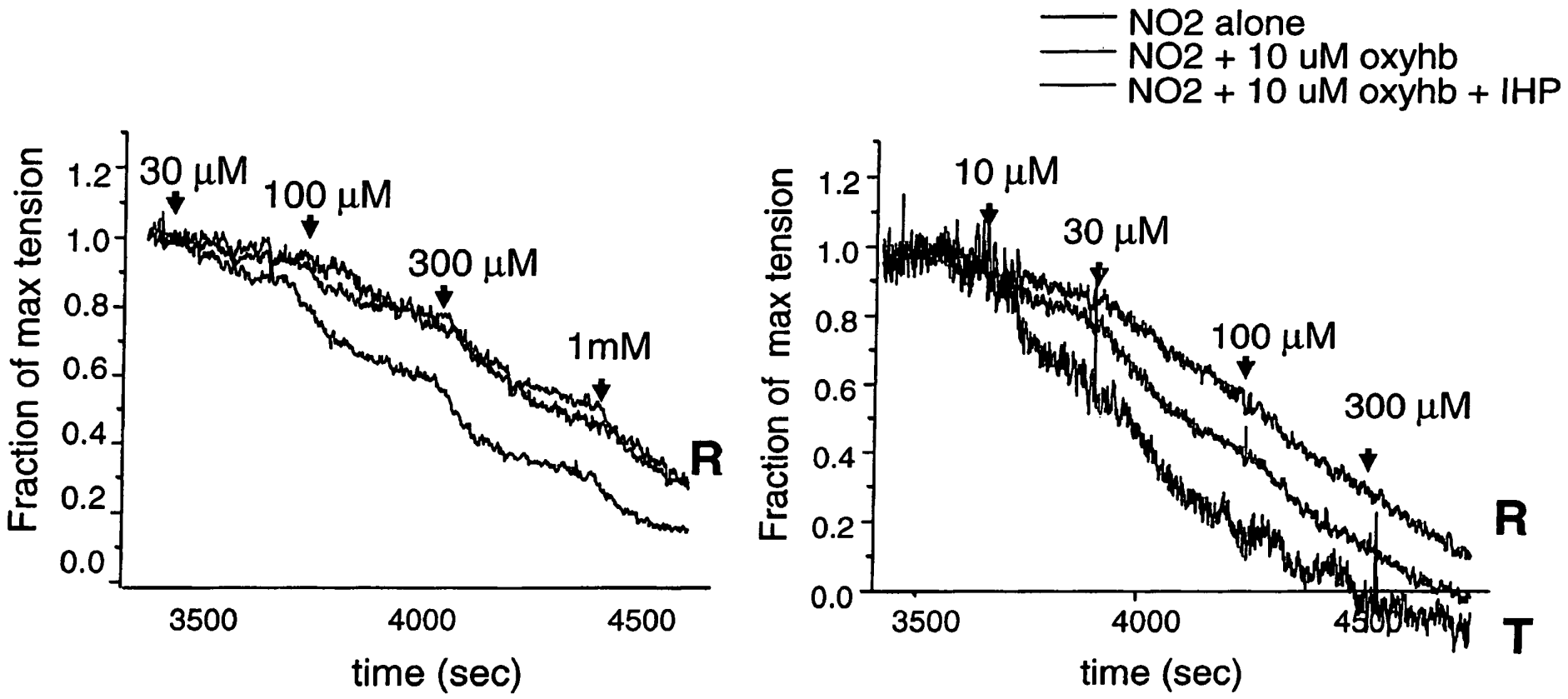


Fig. 25 **Crawford, Gladwin, Patel. Unpublished observations**

Exhaled NO During Nitrite Perfusion in Isolated Rat Lung

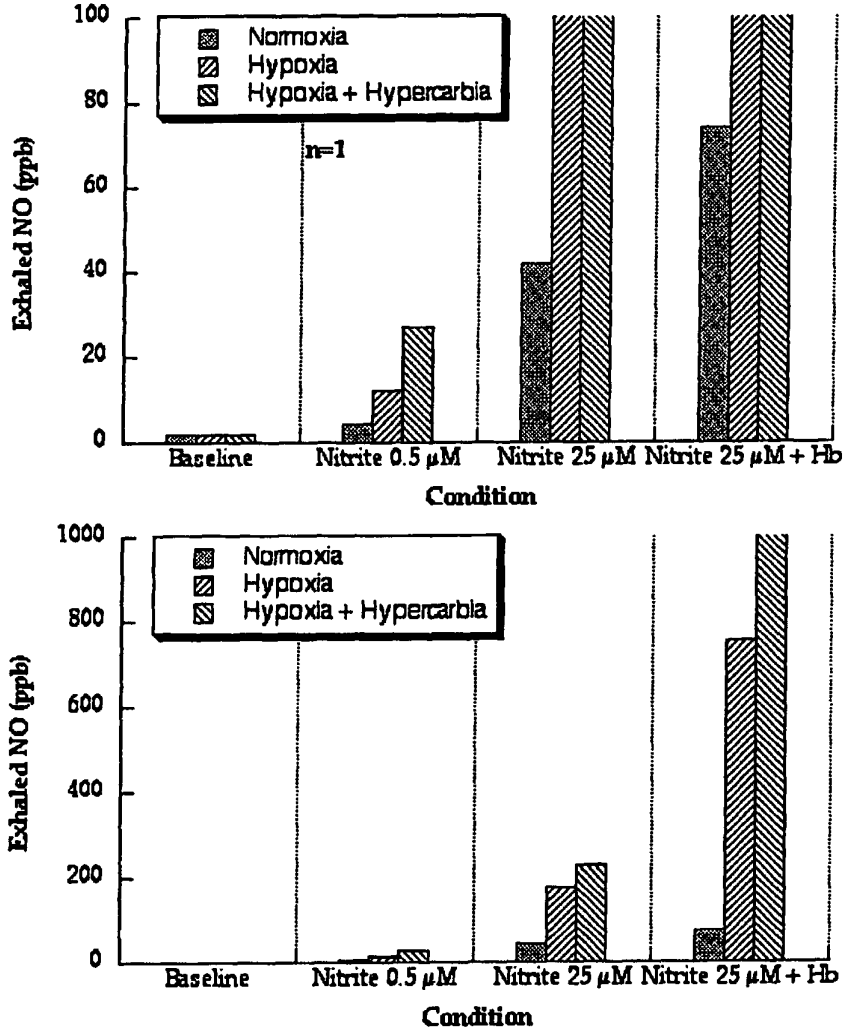


Fig. 26

Deem, Gladwin. Unpublished observations